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Letters to the Editors

FATTY ACID METABOLISM

IV. KETONE BODIES AS INTERMEDIATES OF ACETATE OXIDATION IN ANIMAL TISSUES[*]

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Recent experiments with isotopically labeled fatty acids (2–6) make it ,reasonably certain that ketone bodies arise from these substances in liver by coupling of acetyl groups which result from β oxidation. With the establishment of the acetyl group as the most probable primary breakdown product of fatty acids, the question emerges whether condensation to acetoacetate is an obligatory step in the metabolism of acetate in all tissues and under all conditions, or whether further chemical changes may occur without intermediary conversion to ketone bodies. The present investigation was designed to elucidate this question.

A preliminary study disclosed that liver, kidney, and heart muscle slices of the rat readily metabolize acetate,[1] but only with liver is there accumulation of ketone bodies. Comparison of the relative rates of acetate and acetoacetate disappearance in these organs revealed that whereas in liver there was little if any disappearance of ketone bodies, the rate of acetoacetate breakdown in kidney and heart is so rapid compared with acetate metabolism, that ketone bodies, if formed from the latter, would not be expected to accumulate. Thus, no answer can be acquired by this method as to whether or not ketone bodies are actually formed during acetate metabolism in kidney and heart.

With sodium acetate, marked by the presence of excess C^{13} in the carboxyl carbon, the conversion of acetate to acetoacetate in these two tissues was tested in the following way. A mixture of isotopic acetate and normal, non-isotopic acetoacetate was incubated with the tissue for 2 hours in oxygen at 38°, the quantity of acetoacetate taken being such as to allow the recovery of an amount sufficient for isotope analysis at the close of the

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[1] Experiments were also carried out with rat brain and skeletal muscle slices, but acetate utilization was so low in these tissues that further work with them appeared fruitless.

experiment.   Under these circumstances any isotopic acetoacetate formed from acetate should mix with the non-isotopic acetoacetate already present and increase its C^{13} content.   Thus, the presence of excess C^{13} in the recovered acetoacetate may be considered proof of its formation from acetate.

<div align="center">EXPERIMENTAL</div>

The labeled sodium acetate was prepared by the Grignard reaction from isotopic CO_2; it contained 3.90 atom per cent C^{13} excess, representing 7.80 per cent C^{13} excess in the carboxyl carbon.   The sodium acetoacetate was prepared from the methyl ester by the procedure of Ljunggren (7).   The substrates were used in concentrations of approximately 0.01 and 0.005 M respectively, in a 0.01 M phosphate-buffered solution of the following composition: $CaCl_2$ 1 mM, KCl 2 mM, NaCl 13.5 mM per 100 ml.

Organs of adult rats, previously fasted 24 hours, were used.   Slices were prepared in the conventional manner.   The kidney preparation used in Experiment 3 was prepared by means of a small press of our own design, which operates by forcing the undiluted tissue, by hand screw pressure, through a finely perforated die.   Microscopic examination of stained smears of such preparations has revealed the absence of intact cells, though apparently undamaged nuclei were present.

Except where indicated otherwise in the descriptions of the individual experiments, the incubation, isolation, and analytical procedures were the same as employed previously (4).

<div align="center">*Results*</div>

Acetate and Acetoacetate Disappearance—The relative rates of acetate and acetoacetate metabolism in various organs of fasted rats are compared in Table I.   The values given are the conventional Q designation (microliters per mg. of dry tissue per hour, the dry weight being estimated as one-fifth of the wet tissue).   They are the average results of many experiments carried out essentially as described in a previous report (4).   In each case, acetate and acetoacetate were determined by direct assay before and after incubation with the tissue slices.   Of the three organs, liver metabolized acetate most rapidly, this being the only organ in which ketone bodies accumulated.   With acetoacetate as substrate, the low rate of its disappearance was accounted for entirely by hydroxybutyrate formation.   With kidney and heart, the rate of acetate disappearance was lower than in liver.   However, there was a high rate of acetoacetate metabolism in these tissues, accounted for only to a small extent by reduction to hydroxybutyrate.

Similar results were obtained by incubation of kidney and heart slices with mixtures of acetate and acetoacetate.   When both substrates were

present, acetoacetate disappeared much more rapidly than acetate. In view of the high rate of acetoacetate metabolism compared with acetate in these tissues the non-accumulation of ketone bodies cannot be considered as evidence for their lack of formation.

TABLE I

Q Values for Acetate and Acetoacetate Disappearance in Tissue Slices of Fasted Rats

Approximately 2 gm. of fresh tissue in 60 ml. of Ringer-phosphate. Experiments, 2 hours in O_2 at 37.5°.

	Substrate						
	Acetate, 0.01 M		Acetoacetate, 0.005 M		Acetate, 0.01 M + acetoacetate, 0.005 M		
	Acetate	Ketone bodies	Aceto-acetate	Hydroxy-butyrate	Acetate	Aceto-acetate	Hydroxy-butyrate
Liver..........	−5.0	2.5	−1.2	1.2			
Kidney........	−4.2	0	−5.3	1.3	−3.0	−4.4	2.0
Heart muscle...	−2.5	0	−3.7	1.1	−1.3	−4.0	1.2

TABLE II

Distribution of C^{13} in Products of Incubation of Acetate-Acetoacetate Mixtures with Rat Kidney and Heart

2 hours in O_2 at 37.5°.   Acetate, 0.01 M; acetoacetate, 0.005 M.

	Experiment 1, kidney slices, 2.44 gm.		Experiment 2, kidney slices, 2.28 gm. plus 0.54 mM NaHC¹³O₃		Experiment 3, kidney brei, 3.0 gm.		Experiment 4, heart slices, 1.80 gm.	
	mM	C^{13} per cent	mM	C^{13} per cent	mM	C^{13} per cent	mM	C^{13} per cent
Acetate utilized	0.0918	3.90	0.0669	0	0.117	3.90	0.043	3.90
Acetoacetate, start	0.297		0.314		0.291		0.276	
β-Carbon		0		0		0		0
Carboxyl carbon		0.05				0.03		
Acetoacetate, recovered	0.140		0.154		0.167		0.149	
β-Carbon		0.81		0.15		0.69		0
Carboxyl carbon		0.73		0.08		0.85		0.06
		0.92						
Hydroxybutyrate	0.071		0.061		0.082		0.039	
β-Carbon		0.60		0.15		0.57		0
Respiratory CO_2	0.539	0.88	1.207*	2.17	0.698	0.60	0.436	0.33

* Respiratory CO_2 plus CO_2 from the added bicarbonate.

Ketone Body Formation from Acetate by Kidney Slices, by Use of Isotopic Acetate—The data of a single experiment, representative of four thus far carried out, in which a mixture of carboxyl-labeled acetate and non-isotopic acetoacetate was incubated with kidney slices, are listed under Experi-

ment 1 of Table II. The acetone isolated by decarboxylation of the aceto-acetate remaining at the close of this experiment had an excess of 0.27 per cent C^{13}. To ascertain the distribution of the isotope among the 3 acetone carbon atoms, a portion was treated with alkaline hypoiodite, and the resulting iodoform analyzed for its C^{13} content. It was found to contain only the normal C^{13} concentration. Inasmuch as the iodoform carbon arises from the methyl groups of acetone, the excess C^{13} of the acetone must have been exclusively in the carbonyl group. Hence, the C^{13} content of the carbonyl carbon (the β-carbon of acetoacetate) is $0.27 \times 3 = 0.81$ per cent. Two values were obtained for the C^{13} excess of the carboxyl carbon: 0.73 by thermal decarboxylation and 0.92 by aniline citrate decarboxyla-tion. Inasmuch as the latter procedure is more specific for carboxyl carbon of β-keto acids, it can be regarded as the more reliable figure.

There was also recovered 0.071 mM of hydroxybutyrate, which, upon oxidation with dichromate, yielded acetone with a C^{13} excess of 0.20 per cent, representing 0.60 per cent in the β-carbon.

The conversion of acetate to acetoacetate and hydroxybutyrate in kidney thus appears to be established. The presence of the excess isotope in approximately equal concentration in the β-carbon and the carboxyl carbon atoms and its absence from the α- and γ-carbons suggest that the conver-sion occurs by coupling of acetyl groups. However, several other possibili-ties remained to be considered. The remote possibility of a non-enzymatic interchange between acetate and acetoacetate is eliminated for the follow-ing reason. The initial C^{13} values for the acetoacetate were obtained by isotopic analysis of the acetone and CO_2 resulting from heat treatment of an aliquot of the medium before incubation. Inasmuch as the medium contained the isotopic acetate in addition to the non-isotopic acetoacetate, the acetone and CO_2 should have had a C^{13} excess if interchange occurred. This procedure was followed in many experiments, but in no case did the isolated acetone contain excess C^{13}. The slight excess of C^{13} in the carboxyl group remains unexplained. However, it can conceivably be due to a slight decarboxylation of acetate under the conditions employed, since only about 0.3 per cent decarboxylation of the acetate would account for the observed C^{13} excess in the acetoacetate carboxyl carbon.

The possibility remained that the excess C^{13} in the acetoacetate arose indirectly from acetate by a mechanism involving assimilation of isotopic CO_2 resulting from acetate oxidation. Accordingly, an experiment was made with non-isotopic substrates, to which was added 0.5 mM of isotopic sodium bicarbonate containing 4.5 per cent C^{13} excess (Experiment 2, Table II). To keep the CO_2 content of the medium as high as possible the KOH papers usually placed in the central well of the respirometer flasks were omitted. The slight C^{13} excess observed in both the aceto-acetate and hydroxybutyrate fractions suggests that CO_2 assimilation may

play a part in ketone body formation. This reaction is being studied further. It is evident, however, that CO_2 assimilation cannot be responsible for the results obtained with isotopic acetate, since the C^{13} excess in the ketone bodies with isotopic acetate was considerably higher than with isotopic bicarbonate despite the fact that the C^{13} content of the respiratory CO_2 with acetate, 0.88 per cent, was only about one-fourth the average C^{13} content of the respiratory CO_2, $(4.5 + 2.2)/2 = 3.4$ per cent, with isotopic bicarbonate.

Ketone Bodies from Acetate in Pulped Kidney Tissue—In a study of acetate oxidation by animal tissue Wieland and Jennen (8) found acetate to be oxidized readily by cattle kidney brei. In confirmation, we found that rat kidney brei also oxidized acetate at about the same rate as slices, and also without accumulation of ketone bodies. It was of interest, therefore, to compare the course of acetate metabolism in brei with that in slices. Typical results of three such experiments are shown under Experiment 3 of Table II. They indicate that acetate metabolism in this preparation is qualitatively and quantitatively similar to that with slices.

Acetate Oxidation in Heart Muscle—By contrast with kidney, acetate metabolism in rat heart slices apparently does not proceed through ketone bodies. As shown in Experiment 4 of Table II, the β-carbons of acetoacetate and hydroxybutyrate contained no excess C^{13}, despite the fact that acetate oxidation had doubtlessly occurred, as indicated by the presence of 0.33 per cent excess C^{13} in the respiratory CO_2.

Calculation of Quantity of Acetate Converted to Ketone Bodies—With the establishment of ketone body formation from acetate by kidney, the question arises as to what percentage of the total acetate metabolized was thus transformed. In Experiment 1, the excess of 0.81 per cent C^{13} in the β-carbon atom represents an over-all C^{13} excess of 0.41 atom per cent in the acetoacetate (assuming equal quantities in the β-carbon and carboxyl carbon and none in the α- and γ-carbons). Inasmuch as the acetate contained 3.90 atom per cent excess C^{13}, of the 0.211 mM of ketone bodies recovered $(0.41 \times 0.211)/3.90 = 0.022$ mM came from acetate, requiring the condensation of $0.022 \times 2 = 0.044$ mM of acetate, or $(0.044 \times 100)/0.092 = 48$ per cent of the total which disappeared. This method of calculation, however, yields only a minimum value, since no account is taken of the isotopic acetoacetate metabolized. A more accurate though still approximate calculation can be made, the isotopic acetoacetate which disappeared being considered, by using the equation derived below.

We may consider the conversion of acetate to acetoacetate, and the concurrent disappearance of the latter, as part of a series of consecutive reactions of the type

$$A \rightarrow B \rightarrow C$$

where A and B are acetate and acetoacetate, respectively.

Let R_1 = the rate of formation of B from A, assumed to remain constant. R_2 = the rate of disappearance of B, assumed to remain constant and different from R_1. B^* = the quantity of B derived from A, present in the total B at any time, t.[2] B_0 = the initial quantity of B.

The quantity of B present at any time t is a function of t, equal to $B_0 + (R_1 - R_2)t$. At any instant, the rate of formation of B^* will be equal to R_1 and the rate of disappearance of B^* will equal $R_2B^*/[B_0 + (R_1 - R_2)t]$. The rate of change of B^* will then be equal to its rate of formation minus its rate of disappearance.

$$(1) \qquad \frac{dB^*}{dt} = R_1 - \frac{R_2 B^*}{B_0 + (R_1 - R_2)t}$$

Integration of Equation 1, considering that $B^* = 0$ when $t = 0$, yields[3]

$$(2) \qquad B^* = B_0 + (R_1 - R_2)t - B_0^{\frac{R_1}{R_1-R_2}} [B_0 + (R_1 - R_2)t]^{-\frac{R_2}{R_1-R_2}}$$

Since $B^* = i_B [B_0 + (R_1 - R_2)t]/i_A$ where i_B and i_A are the isotope contents of B and A, respectively, we may substitute this value for B^* in Equation 2, yielding

$$(3) \qquad \frac{i_B}{i_A} = 1 - \left[\frac{B_0}{B_0 + (R_1 - R_2)t}\right]^{\frac{R_1}{R_1-R_2}}$$

Solving Equation 3 for R_1 gives

$$(4) \qquad R_1 = \frac{(R_2 - R_1) \log \dfrac{i_A}{i_A - i_B}}{\log \dfrac{B_0}{B_0 + (R_1 - R_2)t}}$$

In Equation 4, all the values on the right side are experimentally determinable, except $R_2 - R_1$, which is easily derived from the initial and final values of B. Since $[B_0 + (R_1 - R_2)t]$ is equal to the final value of B, $R_2 - R_1 = -(R_1 - R_2) = (B_0 - B \text{ final})/t$. It will be positive if B decreases and negative if B increases.

Equation 4 thus allows the calculation of the rate of formation of an intermediate from an isotopically labeled precursor regardless of the degree of variation in the total quantity of the intermediate, provided its initial and

[2] Since the B molecules formed from A will have the same isotope concentration as A, it follows that $B^*/B = i_B/i_A$. A numerical example will make this clear. If A contains 10 per cent excess isotope and 100 mM of B are isolated, having 5 per cent excess, the proportion of the total B derived from A will be 5/10 and $B^* = 5 \times (100/10) = 50$ mM.

[3] We are indebted to Dr. G. A. Mills of the Houdry Laboratories for aid in the integration of this equation.

final values can be determined. It is not applicable, however, when R_1 and R_2 are nearly equal, as under these circumstances both numerator and denominator approach zero and the equation becomes indeterminate. If R_1 and R_2 are equal or nearly so, B may be assumed to have a constant value; the same reasoning used in the derivation of Equation 4 then leads to

(5) $$R = R_1 = R_2 = \frac{2.303B \log \dfrac{i_A}{i_A - i_B}}{t}$$

When the data of the kidney slice experiment are applied to Equation 4, in which $i_A = 3.90$, $i_B = 0.41$, $B_0 = 0.297$, B final $= B_0 + (R_1 - R_2)t = 0.211$, $R_2 - R_1 = 0.043$, and $t = 2$, there is obtained a value for R_1 of 0.0140 mM per hour, or 0.0280 mM of acetoacetate formed from acetate during the 2 hour incubation period. Thus, 0.056 mM of acetate, or $(0.056 \times 100)/0.0918 = 61$ per cent of the total utilized was converted to ketone bodies.

Of three experiments with kidney slices, the proportions of the total acetate utilized involved in ketone body formation were 36, 60, and 61 per cent, respectively; and of two experiments with kidney brei, 51 and 43 per cent of the total acetate had formed ketone bodies.

It should be emphasized that any method of calculation based on experiments in systems of the types described are necessarily subject to great uncertainties; therefore the figures obtained can only be considered as rough approximations. Probably the most serious uncertainty is in the extent to which added substrates reach equilibrium with intracellular components. Inasmuch as we obtained quantitatively similar results with slices, in which the cells are largely intact, and in pulps in which cellular organization was to a great extent disrupted, it would appear that cellular permeability was not an important factor in our results. The possibility remains, however, that other, as yet undisclosed, factors prevent attainment of complete equilibrium. Until such effects can be evaluated, we can only assume that the figures given represent a reliable measure of the order of magnitude of the conversion. The results of all the experiments together indicate that about one-half of the total acetate was metabolized without condensation to ketone bodies.

That not all of the acetate utilized formed ketone bodies is indicated also by comparison of the C^{13} content of the respiratory CO_2 with that of the recovered acetoacetate. In Experiment 1, for example, the respiratory CO_2 had a C^{13} excess of 0.88 per cent as compared with 0.41 atom per cent for the acetoacetate. In Experiment 3, the respective values are 0.60 and 0.35 per cent. If all of the acetate were converted to ketone bodies, the C^{13} content of the respiratory CO_2 would reflect the average C^{13} concentra-

tion of the acetoacetate during the course of the incubation, which would be about one-half of its final value. Inasmuch as the respiratory CO_2 had a C^{13} excess of 3 to 4 times this value, the conclusion seems warranted that part of the acetate is metabolized in kidney by a pathway not involving ketone body formation.

In the experiments with heart muscle, no evidence of the formation of ketone bodies was observed. With a precision of ± 0.02 per cent C^{13} an excess of 0.04 per cent in the acetone would have been detectable, representing an over-all C^{13} content of 0.06 atom per cent in the acetoacetate. By Equation 4, this would require the conversion of 0.006 mM of acetate or about 15 per cent of the quantity utilized in the experiment. Thus, if conversion to acetoacetate occurred, it must have been less than 15 per cent.

DISCUSSION

The observation that only part of the acetate utilized by kidney and none of that utilized by heart is converted to ketone bodies indicates that these substances are not obligatory intermediates of acetate oxidation. However, inasmuch as condensation to acetoacetate was found to be a major metabolic reaction of acetate in kidney, it appears that ketone body formation may occur more generally than was hitherto realized. To this extent existing concepts of ketosis, based on the principle that ketone body formation is a special mechanism of fat metabolism, occurring exclusively in liver (9, 10), require revision.

Although the old concept of a coupling of fat and carbohydrate metabolism, exemplified in the familiar expression, "fats burn in the flame of carbohydrates" fell into disrepute in recent years (9–11), there now exists a body of circumstantial evidence indicating that the metabolism of fatty acids whether at the acetate or acetoacetate level, is coupled with a carbohydrate intermediate, which may be oxalacetate.

This evidence may be summarized briefly as follows: (a) Various studies suggest that in the Krebs cycle formulated for carbohydrate oxidation the substance undergoing reaction with oxalacetate is not pyruvate, but an acetyl derivative formed by oxidative decarboxylation of pyruvate (11–13). (b) Other investigations indicate that acetate is oxidized via the Krebs cycle in yeast (14–16), and the recent report of Rittenberg and Bloch (17) suggests a similar mechanism in animal tissues. (c) The conclusion of Breusch (18) and Wieland and Rosenthal (19) that acetoacetate is metabolized by animal tissues via the Krebs cycle, though disputed by Krebs and Eggleston (20), was confirmed recently by the isotope tracer experiments of Buchanan et al. (21).

The studies referred to above constitute a basis for the belief that acetate, pyruvate, and acetoacetate are metabolized by a common pathway involving as intermediates components of the Krebs cycle. It is therefore

conceivable that all three are convertible to a common intermediate which may be acetyl phosphate (13) or a "ketene-like" substance, as suggested by Martius (13). The condensation of "active" acetate with oxalacetate and thence through the Krebs or a similar cycle thus provides a common pathway for the complete oxidation of fatty acids and carbohydrates, as well as certain amino acids, in so far as they can yield acetyl groups (5, 6). According to this postulation, the ketone bodies are considered to be a transport vehicle for acetyl groups, being formed in tissue lacking in oxalacetate, and carried by the body fluids to others where they are reconverted to acetyl to enter into reaction with oxalacetate. Further details of the mechanism of fatty acid metabolism must await the identification of other intermediates of acetate metabolism in animal tissues. Such studies are now under way.

SUMMARY

With isotopic sodium acetate, it was shown that conversion to ketone bodies is a major metabolic reaction in rat kidney, about 50 per cent of the acetate utilized being thus converted. In rat heart muscle, acetate is oxidized without intermediary conversion to ketone bodies.

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A STUDY OF THE EFFECT OF GROWTH SUBSTRATE ON THE RESPIRATION OF AZOTOBACTER*

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It is well known that the medium upon which bacteria are grown has a definite influence on the properties of the resulting cells. Several investigators (1–3) have reported that the physiological activities of species of *Azotobacter* may be altered by the growth medium. This investigation was undertaken to determine the response of *Azotobacter* cells to different organic compounds available for respiration.

The problem was divided into two parts: (a) growing the organisms on different energy sources and studying their physiological activity to determine whether the substrate had any effect upon the enzymatic constitution of the cells, and (b) determining whether the *Azotobacter* cells could adapt themselves to the utilization of a substrate other than the compound serving as the energy source for the growth of the organisms.

EXPERIMENTAL

All cultures used in these studies were tentatively identified as strains of *Azotobacter chroococcum* Biej. Before a strain was used experimentally its growth characteristics were carefully studied, particular attention being given to the purity of the culture and the stability of its colony characteristics. Cell suspensions were prepared by growing the cultures in an aerated mineral salts medium containing the desired substrate, according to the procedure described by Harris and Gainey (4). When the organisms were grown in media containing the sodium salts of organic acids, the reaction of the medium tended to become alkaline but was maintained near the neutral point of brom-thymol blue by frequent additions of the corresponding organic acids.

Respiration studies were made with the Warburg type micro respirometer with the technique used by Burk and Lineweaver (5). 1 ml. of 0.05 M solution of the specific organic substrate was dumped from the side arm into the flask at the beginning of the experiment. The flask contained from 300 to 500 million *Azotobacter* cells suspended in 0.03 M

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phosphate solution (adjusted to pH 7.0) plus 0.0005 M calcium as CaCl₂. All respiration data herein reported were obtained at 30°. Duplicate or triplicate flasks were employed in all cases. Endogenous respiration was practically nil; hence the error that might result from oxygen uptake by the cells in the absence of added substrate was negligible.

Methylene blue reduction tests for the presence of specific dehydrogenases followed the usual Thunberg technique as described by Quastel and Whetham (6). Visual judgment of complete disappearance of the blue color, used as the criterion, is subject to large error, but the differences in the rates of reduction were of such an order that there can be no doubt regarding their significance.

TABLE I

Summary of Respiration of Cultures Grown on Mannitol and Glucose

Strain of *Azotobacter*	Cultures grown on	O₂ utilized during initial 30 min. in presence of	
		Glucose	Mannitol
		c.mm.	*c.mm.*
10b	Mannitol	16	74
10b		19	125
10b		17	119
44		18	70
60		21	89
60		13	76
10b	Glucose	52	9
10b		58	10
44		53	10
60		79	6
60		116	3
60		74	11
60		130	8

Effect of Growth Substrates upon Respiratory Activity—The organic compound serving as the energy source for *Azotobacter* during the growth of the cells will be referred to as the *growth substrate*. The compounds upon which the physiological activities of the cells were measured in the respirometer and Thunberg experiments will be spoken of as the *respiration substrate*. Since mannitol and glucose have been extensively used for the growth of this organism, and since Nilsson (1) has indicated that the dehydrogenase activity of *Azotobacter* differs in cells grown on these two compounds, respiration studies were carried out with three different strains of the organism grown on glucose or on mannitol. The initial rates of oxygen uptake in the presence of these compounds during the first 30 minutes of the respiration experiments are given in Table I. No

particular effort was taken to insure the same number of cells in the different cell suspensions; therefore the absolute rates of oxygen uptake for the different suspensions cannot be compared directly. Without exception far greater respiratory activity was observed when the cells were oxidizing the compound used as the growth substrate. For the cells grown on mannitol the respiration rate upon glucose was only 13 to 25 per cent of the rate on mannitol. Similarly the cells grown on glucose utilized only 3 to 19 per cent as much oxygen in mannitol solution as when in the presence of glucose.

In order to study further the effect of the growth substrate upon respiration, cultures were grown on media containing gluconate, succinate, lactate, acetate, mannitol, glucose, and ethyl alcohol as the energy source.

TABLE II

Time Required for Reduction of Methylene Blue in Presence of Different Respiratory Substrates by Cells Grown on Various Growth Substrates (Culture 60)

Cells grown on	Time required for reduction of methylene blue in presence of							
	Mannitol	Glucose	Gluconate	Succinate	Lactate	Acetate	Alcohol	Pyruvate
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
Mannitol......	**9**	90	150	180	25	60	210	50
Glucose.......	90	**10**	4	90	4	10	60	13
Gluconate.....	240	90	**7**	240	50	60	150	50
Succinate.....	240	150	240	**25**	35	240	60	60
Lactate.......	240	240	240	240	**5**	35	120	20
Acetate.......	360	360	360	360	120	**50**	180	50
Alcohol........	360	360	360	360	360	360	**25**	120

The values in bold-faced type represent homologous substrates.

The physiological activity of the washed cells was studied by both the respirometer and the methylene blue reduction techniques. In Table II are recorded the times required for the reduction of methylene blue by cells of Culture 60 grown and tested in the presence of the various compounds indicated. Since the suspensions from the different growth substrates varied in the concentration of cells, comparisons should be restricted to horizontal lines. The differences in the dehydrogenase activities of cells from the seven growth substrates are quite marked. With the exception of cells grown on glucose, the most rapid reduction occurred when the hydrogen donor was the same as the growth substrate. Considerable activity, however, was shown in the presence of lactate and acetate, regardless of the growth substrate. Succinate salts did not serve well as hydrogen donors except when the cells were grown on succinate.

The comparative respiration rates of these same cells are given in Table

III. The oxygen uptake in the presence of the growth substrate has been taken as 100 per cent. In all instances, except glucose, the respiration was greater in the presence of the growth or homologous substrate than in the presence of any of the other compounds tested. Again lactate and acetate were readily oxidized by cells grown on heterologous substrates. Curves showing the rate of oxygen utilization by four of these cell suspensions during a 2 hour period are given in Figs. 1 to 4. These graphs show that the compound which served as the growth substrate of the cells has a marked influence on the ability of the organisms to carry on respiration in the presence of various respiration substrates.

TABLE III

Comparative Rates of Oxygen Uptake by Azotobacter Cells First 30 Minutes in Presence of Various Respiratory Substrates

O₂ consumed in presence of homologous substrate taken as 100.

Cells grown on	Relative rate of O₂ uptake in presence of 0.05 M							
	Mannitol	Glucose	Gluconate	Succinate	Lactate	Acetate	Alcohol	Pyruvate
Mannitol......	**100**	17	9	13	68	89	3	57
Glucose.......	3	**100**	131	13	136	80	10	83
Gluconate.....	1	32	**100**	10	56	69	16	63
Succinate.....	0	0	0	**100**	78	0	10	16
Lactate.......	1	1	3	19	**100**	64	10	69
Acetate.......	1	3	1	9	27	**100**	9	47
Alcohol.......	13	14	5	5	6	56	**100**	31

The values in bold-faced type represent homologous substrates.

Thus, both the measurement of oxygen uptake and the ability of the *Azotobacter* cells to reduce methylene blue in the presence of different substrates have shown that the physiological activity depends to a considerable extent upon the compound serving as energy source during the growth of the cells.

In all cases throughout the experiments the *Azotobacter* cells brought about a rapid utilization of the compound which served as the growth substrate. The uniformly high rate of oxygen uptake is shown by the steep slope and almost straight line of the respiration curve. As has already been noted, certain other compounds were sometimes readily used. This is particularly true in the case of cells grown on glucose which utilized gluconate, lactate, and acetate equally as rapidly as they did glucose (Table III). The ability of the cell to begin oxidizing these compounds immediately might indicate that they are intermediates in the dissimilation of the growth substrate.

While cells in the presence of the growth substrate, and certain other compounds, showed initially high rates of O_2 uptake and gave essentially straight line respiration curves, a distinct lag was evident in the respiration curves of cells in the presence of most of the heterologous substrates studied. For example, when the cells were grown on lactate, very little oxygen was utilized in the presence of mannitol, succinate, glucose, or gluconate during the first 30 minutes of the experiment (Table III and Fig. 1). In many instances, however, respiration in the presence of
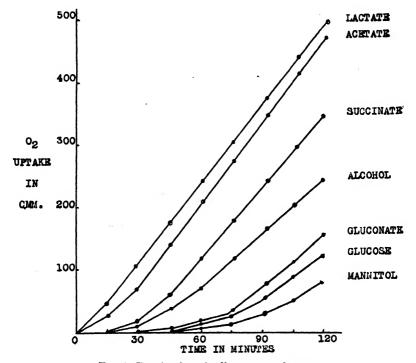
FIG. 1. Respiration of cells grown on lactate

heterologous substrates increased so rapidly that during the third or the fourth 30 minute period the rate of O_2 uptake approximated that in the presence of the homologous compound. This suggests that *Azotobacter* cells may readily adjust their metabolic mechanism to the utilization of new respiration substrates. In order to show this adaptation or increase in the rate of utilization of new respiration compounds, the comparative rates of respiration during the last 30 minutes of the experiment are shown in Table IV. Comparison of these values with those in Table III will reveal the degree of adaptation. Whereas large differences were apparent

between the rate of oxygen uptake on the growth substrate and most other compounds during the initial 30 minutes, these differences became much less, in most instances, when comparisons were made during the final half hour of the 2 hour experimental period.   It should be emphasized that no increase in the number of *Azotobacter* cells nor any measurable increase in size of the cells occurred during any of these short time experi-
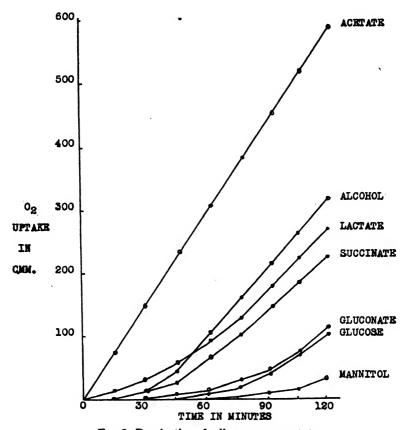
FIG. 2. Respiration of cells grown on acetate

ments, as determined by actual cell counts and microscopic examinations before and after the experiment.

The rate of increase or recovery from the initial lag in respiration varied with the different substrates and with the cells grown on the various energy sources.   The ease with which the cells adapted themselves to a new substrate appears to bear some relation to the complexity of molecules of the new substrate as compared with the growth compound.   Cells

grown on glucose or on mannitol were able to adapt themselves to the utilization of succinate and alcohol quite rapidly. However, when the cells were grown on compounds of lower molecular weight, adaptation to mannitol or glucose did not take place so readily. Cells grown on succinate gave no oxygen uptake on glucose, mannitol, gluconate, or acetate even after 2 hours in the presence of these compounds. An extended period of observation probably would have shown utilization of these compounds, since cells from the succinate suspension grew readily when streaked on
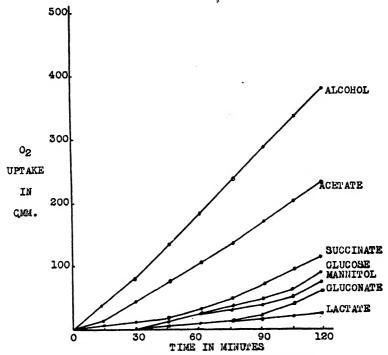
FIG. 3. Respiration of cells grown on ethyl alcohol

mannitol agar plates. Cells grown on all other substrates exhibited more or less adaptation for utilization of the various compounds studied. The data suggest a rapid alteration in the enzymatic systems to make possible oxidation of the new respiration substrate.

In order to determine whether this adaptation was related to the dehydrogenase activity (as measured by the rate of methylene blue reduction), cells grown on one substrate were aerated for a short time in the medium containing a different substrate. No increase in cell numbers occurred during this period. The cells were then thoroughly washed and respiration

studies carried out in the presence of each of the original compounds. In all instances the hydrogen donors, originally only slightly utilized, were readily activated by such aerated cells. Results from two such experiments are presented in Table V. In the first experiment, cells grown on glucose required 90 minutes to reduce methylene blue in the presence of ethyl alcohol and only 4 minutes after having been aerated in the presence of alcohol. The possibility of any appreciable amount of the original or intermediate metabolites being retained with the aerated cells was checked
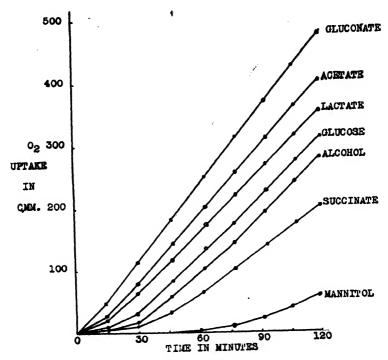
FIG. 4. Respiration of cells grown on gluconate

by the use of controls containing only cells, dye, and buffer. Measurements of oxygen uptake of the same cells employed in the above experiments gave similar results.

These data also show that when *Azotobacter* cells alter their metabolism to make use of a new substrate they do not immediately lose the power of activating the original growth substance. This might indicate the presence of two substrate-activating mechanisms. In the few experiments performed, however, the oxygen uptake by such cells in the presence of mixtures of the two substrates was found to be no greater than in comparable concentrations of either compound alone.

Limited studies of factors influencing the ability of *Azotobacter* to adjust its respiratory system to new substrates indicate that certain conditions which prevent growth also prevent adaptation. Increased hydrogen ion concentration, anaerobiosis, deficiency of calcium, and the presence of cyanide or of dinitrophenol all tended to inhibit adaptation without greatly affecting the original dehydrogenase activity.

TABLE IV

Comparative Rates of Oxygen Uptake during Final 30 Minutes of 2 Hour Respiration Experiments

Cells grown on	Rate of respiration in presence of 0.05 M						
	Mannitol	Glucose	Gluconate	Succinate	Lactate	Alcohol	Pyruvate
Mannitol.......	**100**	105	129	83	117	136	131
Glucose........	51	**100**	102	78	87	72	91
Gluconate......	34	85	**100**	63	85	82	88
Succinate......	10	0	0	**100**	98	65	96
Lactate........	45	60	72	101	**100**	76	111
Acetate........	16	43	46	55	63	76	107
Alcohol........	39	38	35	45	11	**100**	51

The values in bold-faced type represent homologous substrates.
See the note above Table III.

TABLE V

Adaptation As Shown by Methylene Blue Reduction Tests

Experiment No.		Time required for reduction of methylene blue in presence of			
		Glucose	Mannitol	Ethyl alcohol	Cells only
		min.	*min.*	*min.*	*min.*
I. Cells grown on glucose	No treatment	12		90	+180
	Aerated 4 hrs. in alcohol medium	12		4	+150
II. " " "mannitol	No treatment	150	7		+300
	Aerated 2 hrs. in glucose medium	25	10		+300

SUMMARY

The organic compound serving as the growth substrate for *Azotobacter* has marked influence on the subsequent respiratory activities of the resulting cells, as measured by oxygen uptake and reduction of methylene blue in the presence of a number of common respiratory substrates. When placed under favorable conditions, *Azotobacter* cells are capable of adapting

their respiratory system to the utilization of new compounds in relatiy short periods of time, usually less than 2 hours.

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INHIBITION OF ALKALINE SERUM PHOSPHATASE ACTIVITY DURING LIVER DISEASE*

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Studies have been made on the effect of various ions on the activity of the alkaline phosphatases extracted from tissues. The cyanide ion may activate, inhibit, or be without effect on alkaline phosphatase activity, depending on the particular enzyme and the tissue from which it was extracted (1–5). It has been reported that the increase in serum phosphatase activity in dogs with hepatic damage may be inhibited by adding sodium cyanide to the phosphatase substrate (6). In the present paper further observations have been made on the effect of cyanide, fluoride, and magnesium on the serum phosphatase activity of patients with hepatic damage.

Methods

Inorganic serum phosphate and alkaline serum phosphatase were determined according to the method of Bodansky, β-glycerophosphate being used as substrate (7). A Klett-Summerson photoelectric colorimeter was used to determine the phosphate ion. The substrate was also prepared with the following concentration of salts: $MgSO_4$ 0.01 M, NaF 0.01 M, NaCN 0.0001 to 0.1 M. A concentrated solution of the appropriate salt was made in the substrate-buffer mixture, the pH adjusted to 9.2, and then diluted with further substrate to the desired molarity. The venous blood samples of the normal subjects and patients with liver disease were taken during the morning, but not under fasting conditions, since a small amount of food does not significantly alter the phosphatase level.

Results

The effect of cyanide on the serum phosphatase activity of normal subjects was tested first. Concentrations of NaCN from 0.0001 to 0.1 M had only a slight effect on the phosphatase activity of these individuals (Table I), the average reduction with 0.01 M NaCN being 1.4 units. Tests

were then made on patients with known hepatic damage in whom the phosphatase activity ranged from 4.25 to 23.69 units per 100 cc. of serum. When the initial phosphatase value was above normal, increasing the molarity of the NaCN produced a marked decrease in the phosphatase value (Table I). The average reduction of activity with 0.01 M NaCN was 9.7 units. The average reduction in the number of phosphatase

TABLE I

Effect of Various Concentrations of NaCN on Serum Phosphatase Activity

Name	Inorganic phosphorus	NaCN added to phosphatase substrate								
		None	0.0001 M	0.001 M	0.0025 M	0.005 M	0.0075 M	0.01 M	0.05 M	0.1 M
		Phosphatase units per 100 cc. serum								
Normal subjects										
	mg. per 100 cc.									
D. R.	2.99	3.25	3.49		2.84		2.57	2.33	2.77	2.40
L. M.	3.74	5.36	5.34	5.20	3.26	2.21		1.98	2.15	2.12
L. P.	2.90	3.19	2.94			2.11		3.06	2.87	3.06
F. P.	2.93	3.10	3.36		2.98	2.34		1.66	1.52	1.52
V. D.	1.75	3.43	3.36	3.34	3.52	3.18		2.61	2.61	2.98
W. W.	3.02	2.18	2.33	2.03	1.83	2.03	1.75	1.84	1.78	2.23
Patients with hepatic damage										
F.	2.71	4.25	3.94	3.82	1.84	2.26	1.35	2.19	2.12	2.54
E. D.	1.22	7.88	8.52		5.00			2.52	2.77	2.90
McC.	2.77	8.51	8.22	6.39	4.76	3.35	2.36	2.64	2.43	3.00
S. M.	2.12	9.38	9.88	2.58		1.18	2.46	2.39	1.93	2.09
K.	2.74	10.36		9.14		3.72		3.65	3.37	3.79
C.	2.95	10.52			3.79	3.34	1.56	2.33	2.84	3.35
C. K.	1.52	10.77	10.58		6.86		3.44	2.32	2.56	1.84
M. K.	3.80	14.39	15.50	12.20	7.21	4.03	4.55	4.34	4.96	4.96
DeN.	1.94	16.47		8.80		3.36		2.70	2.19	2.28
A. J.	2.21	16.53	15.77		7.03	5.62	4.77	4.24	3.15	3.02
DeN.	2.34	20.37	19.90	11.72	6.84		4.93	4.46	4.92	4.52
A. J.	2.52	23.69		10.12		4.64	3.92	2.75	2.26	3.38

units varied with the initial serum level, and was greater when the original level was higher. Further, the NaCN inhibited the phosphatase activity to values that are essentially normal. As the concentration of the NaCN was increased, the phosphatase activity was reduced to normal values, *but did not go below normal* even when concentrations of 0.0075 M or higher were employed. Maximum inhibition was obtained between 0.0025 and 0.0075 M NaCN in most cases. Inasmuch as this activity is inhibited by NaCN, it would seem that the increased serum phosphatase activity in

these patients with hepatic disease is chiefly due to an alkaline phosphatase that is sensitive to NaCN.

In the normal subjects $MgSO_4$ (0.01 M) had a slight activating effect on serum phosphatase activity. NaF was without any significant effect. The inhibition produced by 0.01 M NaCN was also observed in the presence of Mg (Table II). In the patients with hepatic damage Mg also had a slight activating effect on phosphatase activity, although in two cases inhibition was observed. No constant effect of NaF was observed in these patients, but in general a slight inhibition was produced. The

TABLE II

Effect of Cyanide, Magnesium, and Fluoride Ions on Serum Phosphatase Activity

Name	Additions to phosphatase substrate					
	None	0.01 M Mg	0.01 M NaCN	0.01 M Mg, 0.01 M NaCN	0.01 M F	0.01 M Mg, 0.01 M F
	Phosphatase units per 100 cc. serum					
Normal subjects						
D. R.	3.25	4.22	2.33	2.40	3.85	4.00
L. M.	5.36	7.51	1.98	2.02	4.00	3.70
L. P.	3.19	4.49	3.06	3.31	3.70	4.21
F. P.	3.10	5.14	2.66	2.72	2.53	4.72
V. D.	3.43	5.17	2.61	2.84	3.06	3.98
W. W.	2.18	3.19	1.84	1.79	2.26	3.31
Patients with hepatic damage						
A. J.	23.69	15.62	2.75	4.16	21.67	27.22
K.	10.36	11.37	3.65	4.14	9.65	10.66
DeN.	16.47	20.25	2.70	3.50	19.08	19.21
M. K.	14.36	15.69	4.34	4.29	14.76	15.87
DeN.	20.37	23.21	4.46	3.33	16.25	22.27
A. J.	16.53	17.00	4.24	3.88	15.77	15.96
C. K.	10.77	9.80	2.32	2.19	10.20	10.20
E. D.	7.88	9.72	2.52	2.69	7.47	8.95

characteristic inhibition of 0.01 M NaCN was again observed in the presence of Mg (Table II). Magnesium plus fluoride did not produce any noteworthy response.

It seemed possible that when serum was added to the substrate containing NaCN some of the substrate might be hydrolyzed before sufficient time had elapsed for complete inhibition of the phosphatase. In order to test this possibility serum was incubated with NaCN before the substrate was added. A 0.1 M NaCN solution was adjusted to pH 9.2 without buffer or substrate. This solution was diluted with distilled water to

the desired molarity. 0.5 cc. of the NaCN was incubated with 0.5 cc. of serum for 10 minutes at 37°, the final molarity of the NaCN being indicated in Table III. After 10 minutes of incubation 4.5 cc. of buffered substrate, containing the same concentration of NaCN, were added and hydrolysis carried out for 1 hour. The inhibition observed in control subjects is not significantly different from the standard procedure in Table

TABLE III

Alkaline Serum Phosphatase after Preincubation with NaCN

Name	Inorganic phosphorus	Concentration of NaCN added					
		None	0.0001 M	0.001 M	0.005 M	0.01 M	0.05 M
		Phosphatase units after preincubation for 10 min.					
Normal subjects							
	mg. per 100 cc.						
T. L.	2.87	2.49	1.43	1.02	0.68	0.83	0.43
MacC.	3.87	2.03	1.73	1.48	1.58	1.58	1.61
M. M.	1.60	3.20	3.00	1.20	0.90	1.25	1.45
V. D.	2.90	2.60	2.45	2.05	1.85	1.95	1.70
N. H.	2.87	1.78	1.33	0.63	0.78	0.53	0.43
Patients with hepatic damage							
H. P.	1.85	3.65	4.00	2.25	3.30	2.70	2.65
J. M.	1.25	4.65	5.20	2.75	1.45	1.70	1.80
"	2.50	5.25	5.50	5.45	2.20	1.55	1.80
H. S.	3.25	6.40	5.75	3.10	1.60	0.90	1.10
H. B.	3.45	6.45	6.55	6.05	5.30	1.55	1.25
A. T.	2.37	6.88	7.38	8.63	6.38	2.38	3.03
A. M.	3.62	7.23	7.83	7.38		5.01	2.23
S.	3.83	8.28	8.23	9.13	8.27	2.13	1.43
G. F.	4.00	8.30	8.50	8.75	5.00	1.50	1.25
S. M.	2.25	8.75	8.95	2.50	2.80	3.00	1.05
A. J.	2.87	13.93	12.33	7.03	3.03	2.78	2.03
J. M.	1.72	14.03	16.53	15.78	11.28	4.43	4.03

I, and the findings by this technique (Table III) confirmed the conclusions drawn from Table I.

DISCUSSION

In patients with hepatic damage and serum phosphatase values above normal, the addition of NaCN to the substrate inhibited phosphatase activity to essentially normal values. The increase in serum phosphatase during hepatic damage cannot be due to a hexosediphosphatase, as this enzyme will not act on β-glycerophosphate, and, further, it is activated by cyanide (5). Cloetens (4) has prepared extracts of liver tissue which

he fractionated into alkaline phosphatases I and II. Alkaline phosphatase I was not inhibited by 0.01 M KCN but its activity was reduced by 0.01 M KF. Alkaline phosphatase II was unaffected by 0.01 M KF but was completely inhibited by 0.01 M KCN. In the present study the effect obtained with NaCN and NaF on the serum phosphatase activity would suggest that the elevation of phosphatase in serum from patients with hepatic damage may be due to an enzyme corresponding to Cloetens' alkaline phosphatase II. The same results were obtained in dogs with liver damage (6).

The effect of NaCN does not depend on the initial concentration of phosphatase in the serum. The same effect of increasing concentrations of NaCN was obtained when the initial values of phosphatase ranged from slightly above normal to as high as 23.69 units per 100 cc. of serum. This was further checked by diluting some of the sera with saline before incubating with NaCN. When this was done, the relative amount of inhibition was the same as in the undiluted serum. The results were also independent of the type of liver damage. Provided the initial concentration of serum phosphatase was above normal, NaCN produced the same characteristic effect.

SUMMARY

A concentration of 0.01 M $MgSO_4$ had a slight stimulating effect on the alkaline phosphatase activity of serum from normal subjects and from patients with hepatic damage. Sodium fluoride (0.01 M) was without any marked or consistent effect.

Concentrations of NaCN from 0.0001 to 0.1 M had a small inhibitory effect, averaging 1.4 units, on the serum phosphatase activity of normal individuals. In patients with high serum phosphatase values due to liver damage the addition of NaCN to the substrate inhibited the increased phosphatase activity, returning the values to normal levels. With increasing concentrations of NaCN the serum phosphatase activity decreased to normal values, but the values did not go below normal. It is suggested that the increase in alkaline serum phosphatase observed during liver damage is due to a phosphatase or phosphatases that are sensitive to NaCN even in the presence of magnesium.

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ANALYTICAL DETERMINATION AND SOME PROPERTIES OF SEVERAL THYROID-INHIBITING COMPOUNDS AND OF SUBSTANCES RELATED TO THEM

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Of the large number of substances studied by Astwood (1) and by Astwood, Bissell, and Hughes[1] with regard to their inhibitory effects upon the thyroid gland, a number were found to be more effective than was 2-thiouracil[1] in rats. Most of these are substituted 2-thiouracils (2), the substituent being a hydrocarbon group in either the 5 or 6 position, or in both, substitution in the 6 position yielding the more active compounds. On the other hand if the substituent in the 6 position was an amino or a carboxyl group, the activity of thiouracil was lost. Also among the highly active compounds were the dissimilar structures, 5,5-diethyl-2-thiobarbituric acid, 2-mercapto-5-amino-1,3,4-thiadiazole, and 2-mercaptoimidazole.

This communication reports the results of the application of analytical procedures to a number of active sulfur compounds and related inactive substances, and a study of the ultrafiltrability of these compounds when added to serum, by techniques previously applied to thiouracil (3).

EXPERIMENTAL

The compounds studied were crystalline preparations, in most cases supplied through the kindness of Dr. E. B. Astwood. Thiourea and thiobarbituric acid were products of the Eastman Kodak Company. N-Acetyl- and N,N'-diacetylthiourea were prepared according to Werner (4). All of these substances were used in the form of freshly prepared solutions containing 1 mg. per ml., prepared by the addition of an equivalent or less of alkali except in the case of the acyclic compounds and of dihydrothiouracil and 2-mercaptoimidazole, which dissolved readily in water without alkali addition.

Study of Analytical Procedures for These Compounds—The 6-substituted 2-thiouracils could be determined in serum ultrafiltrates or in cerebrospinal fluid by the application of a modified Grote's reagent, as described for 2-thiouracil (3). Study of the absorption spectra of 2-thiouracil, 6-*n*-propyl-2-thiouracil, and 6-benzyl-2-thiouracil indicated that the colorants were very similar although not identical. The relative depths of color produced as measured by the Coleman universal spectrophotometer at

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[1] Astwood, E. B., Bissell, A., and Hughes, A. M., unpublished results.

660 mμ are shown in Table I. These relationships were modified when the observations were made at a greater wave-length. When the substituting group was a hydrocarbon group containing up to 3 carbon atoms, or a carboxyl group, these compounds could be recovered from serum (Table I) by the use of p-toluenesulfonic acid as the deproteinizing agent, as previously described (3).[2] With larger hydrocarbon groups recoveries were incomplete, being less than 50 per cent for 6-phenethylthiouracil. The failure to

TABLE I

Recovery of Derivatives Added to Serum, with Subsequent Deproteinization by
p-Toluenesulfonic Acid

Compound	Relative optical density per mole; 2-thiouracil = 100 at 660 mμ	Serum concentration	Recovery from serum
		mg. per cent	*per cent*
6-Methyl-2-thiouracil....................	88	5.0	98, 91
6-Ethyl-2-thiouracil.....................	113	5.0	93, 99, 88
6-Propyl-2-thiouracil....................	109	5.0	100, 92
		1.0	93, 87
6-Hexyl-2-thiouracil.....................	108	5.0	55, 64
6-Benzyl-2-thiouracil....................	117	5.0	69, 67, 76, 52
6-Phenethyl-2-thiouracil.................	113	5.0	45, 40
6-Carboxy-2-thiouracil...................	87	5.0	95, 98
6-Amino-2-thiouracil.....................	109	4.4	64, 68
Dihydro-2-thiouracil.....................	2–60*	5.0	101, 100
4-Thiouracil............................	*	5.0	102, 97, 98
2-Thiobarbituric acid....................	28*	2.0	95, 104
5,5-Diethyl-2-thiobarbituric acid.........	*	5.0	95, 107, 100
		4.0	92, 98
2-Mercapto-5-amino-1,3,4-thiadiazole......	126*†	5.0	90, 75, 60
2-Mercaptoimidazole..................	48*	4.0	91, 94

* Colors not comparable.

† Compared at 700 mμ.

recover these compounds was not a result of their solubility being exceeded during deproteinization. 6-Aminothiouracil also was not well recovered from serum.

[2] As deproteinizing agent a combination of hydrochloric acid and trichloroacetic acid has been used in place of p-toluenesulfonic acid. To 2 ml. of serum are added 3 ml. of N hydrochloric acid and then 5 ml. of 5 per cent trichloroacetic acid. Filtrates obtained by this procedure have not shown turbidity upon neutralization; hence the use of chloramine-T (3) is omitted. Recoveries by this procedure were satisfactory for the substances tested, thiouracil, 6-propylthiouracil, and 5,5-diethylthiobarbituric acid. (Unpublished results, Christensen and Paul Fenimore Cooper, Jr.)

Dihydro-2-thiouracil showed an unusual behavior with the color reagent. Freshly prepared solutions in water gave a color having an optical density at 660 mμ of only about 2 per cent of that obtained with the same amount of thiouracil. Samples held for some hours at various pH values showed upon subsequent treatment with modified Grote's reagent at pH 8 a depth of color which was related to the pH of the fore period. The greatest color, obtained after storage in 0.1 N sodium hydroxide, was about 60 per cent of that obtained with 2-thiouracil. Analyses could be obtained for dihydrothiouracil by holding standards and unknowns at the same pH (8.0) for 24 hours at room temperature before addition of the color reagent. The transmittance was read at 610 mμ.

The color given by 4-thiouracil with Grote's reagent differed according to whether or not the Grote's reagent was incubated with glycine before use. In the former case a dark brown color was observed with high absorption at wave-lengths of about 500 and 700 mμ, whereas in the latter case a green color similar to that of 2-thiouracil was obtained. The following analytical procedure was adopted. To 5 volumes of a solution containing 4-thiouracil were added 1 volume of a 0.5 M phosphate buffer which upon 1:6 dilution had a pH of 7.2, and 1 volume of a 1:5 dilution of Grote's reagent which had been left at room temperature 30 minutes before use. The dilute Grote's reagent lost much of its color at room temperature, but not as much as when incubated with a glycine solution. Transmittances were read at 700 mμ. Dihydrothiouracil and 4-thiouracil were not lost during deproteinization of serum with p-toluenesulfonic acid.

2-Thiobarbituric acid gave a blue-green color with Grote's reagent, although much less color than the 2-thiouracil derivatives. 5,5-Diethylthiobarbituric acid, on the other hand, gave a brown color with a maximum absorption slightly below 500 mμ. The depth of this color was sensitive to small pH differences, the absorption being greater at lower pH. When developed at pH 5 to 6, a deep salmon color was obtained, which appeared and faded rapidly. The analytical procedure adopted was the same as for 4-thiouracil except for three points: as diluent for Grote's reagent, 0.1 M glycine was used instead of water, the diluted Grote's reagent was incubated at 38° instead of at room temperature, and the transmittance was determined at 500 mμ. This modified procedure also gave good results with 2-thiouracil and its derivatives. Pentothal gave a blue-green color of low intensity. Both thiobarbituric acid and its diethyl derivative were recovered readily from serum after deproteinization by p-toluenesulfonic acid.

2-Mercapto-5-amino-1,3,4-thiadiazole could be determined by the procedure described for 2-thiouracil, although the absorption spectra of the pigments were different. The transmittance was read in this case at 700 mμ. Recoveries from serum were not complete for this agent (Table I).

2-Mercaptoimidazole also was readily determined by use of the modified Grote's reagent, and was not lost during deproteinization of serum by

TABLE II

Ultrafiltrability of Derivatives in Serum

	Thyroid effect found by Astwood et al.;* 2-thiouracil = 1	Source of serum	Concentration	pH, 25°	Per cent ultra-filtered
			mg. per 100 gm. H₂O		
6-Methyl-2-thiouracil...............	1.0	Pooled	5.30	7.52	35
		H. N. C.	2.55	7.45	22
6-Ethyl-2-thiouracil.................	7	"	5.35	7.54	13
		"	3.05	7.53	6
6-n-Propyl-2-thiouracil	11	Pooled	10.5	7.51	8
		"	5.26	7.51	5
		"	2.67	7.49	5.6
6-n-Hexyl-2-thiouracil†.............	0.2	H. N. C.	5.25	7.50	0.5
6-Benzyl-2-thiouracil...............	10	Pooled	10.5	7.59	2
		H. N. C.	5.34	7.42	2
		"	3.00	1.92	24
6-Phenethyl-2-thiouracil............	1.2	"	5.40	7.55	2
		Pooled	3.00	7.52	2
6-Carboxy-2-thiouracil..............	0.00	"	5.34	7.50	28
		H. N. C.	3.00	7.55	18
6-Amino-2-thiouracil†...............	0.00	"	4.70	7.42	1
Dihydro-2-thiouracil................	0.1	Pooled	3.07	7.48	85‡
		H. N. C.	5.40	7.57	38§
4-Thiouracil.......................	0.03	"	5.30	7.53	2
		Pooled	3.49	7.56	2
2-Thiobarbituric acid...............	0.00	"	5.30	7.60	65
		H. N. C.	3.06	7.48	55
5,5-Diethyl-2-thiobarbituric acid....	2.0	Pooled	5.00	7.53	44
		"	3.07	7.37	15
2-Mercapto-5-amino-1,3,4-thiadia-zole†..........................	1.6	H. N. C.	5.40	7.54	2
2-Mercaptoimidazole...............	2	"	5.40	7.51	45
		"	3.07	7.50	30

* Effect upon the weight and iodine content of the thyroid glands of rats. Astwood, E. B., Bissell, A., and Hughes, A. M., unpublished results.

† Experiments with similar results are not recorded.

‡ Dihydrothiouracil freshly dissolved.

§ Dihydrothiouracil held at pH 12.5 for preceding 24 hours.

p-toluenesulfonic acid. A comparison of a portion of its absorption spectrum with that of 2-thiouracil is shown in Fig. 1.

By the procedures described above these substances showed satisfactory relationships between concentration and optical density. However, standards were run in each case simultaneously to establish the relationship.

Ultrafiltrability of These Compounds in Human Serum—Ultrafiltration was carried out as described earlier (3) at room temperature, which varied from 22–27°. · In each case a sample of serum containing a similar concentration of the test substance was kept in a tonometer over mercury to simulate the conditions of the ultrafiltration. Recoveries from these samples were either complete or similar to recoveries with freshly mixed serum and sulfur compound. The serum used in these experiments was either a pooled specimen from three or more individuals, or a sample from the same normal donor. The results are recorded in Tables II and III.

TABLE III

Analysis of Serial Samples during Ultrafiltration of Serum Containing Dihydrothiouracil

Experiment No.	Pretreatment of dihydrothiouracil	Period	Original concentration	pH, 25°	Concentration of ultrafiltrate
			mg. per 100 gm. water		*mg. per 100 gm. water*
I	Dissolved in water just before use	1st 6 hrs.	5.35	7.57	3.3
		Subsequent 12 hrs.			2.2
II	24 hrs. at pH 12–13	1st 6 hrs.	5.33	7.35	3.2
		Subsequent 12 hrs.			3.3
III	24 " " " 12–13	1st 6 hrs.	5.35	7.55	2.0
		Subsequent 12 hrs.			2.1

Substitution of a methyl group in position 6 of 2-thiouracil appeared to decrease the binding by proteins, but with larger hydrocarbon groups the binding increased to practically complete in the case of the hexyl and phenethyl derivatives. The solubilities of the hexyl, benzyl, and phenethyl derivatives in 0.1 M phosphate at a final pH of 7.54 at about 25° (6 hours of agitation) were 7.7, 10.5, and 3.3 mg. per cent respectively. Hence the solubilities of these compounds were probably not exceeded, except possibly in one experiment with 6-phenethylthiouracil. The 6-carboxyl group decreased the binding, whereas the 6-amino group produced almost complete combination with proteins.

Electrometric titration of 6-methyl- and 6-propylthiouracil, as previously described for thiouracil (3), indicated pK' = 8.04 and 8.17, respectively. Substitution of alkyl groups at position 6 thus appeared to decrease the dissociation of the mercapto group.

The product of hydrogenation of 2-thiouracil at the 5 and 6 positions,

dihydrothiouracil, showed some inconstancy in ultrafiltrability. When this compound was held in an alkaline solution overnight before being added to serum, the binding was greater than with freshly dissolved dihydrothiouracil. In the latter case, removal and analysis of serial samples of ultrafiltrate during ultrafiltration showed a decreasing concentration. However, if the sulfur compound had first been held in alkaline solution, a steady concentration was observed in the ultrafiltrate (Table III).

The conclusion drawn is that dihydrothiouracil underwent a rearrangement, rather sluggishly at pH 7 to 8, more quickly in more alkaline solution to a form which was bound more strongly by serum proteins. This rearrangement is probably the same as that required for color with Grote's reagent by this compound. That this rearrangement involved the formation of the sulfhydryl group (in either the dissociated or undissociated form) was suggested by the observation that this substance showed delayed neutralization. Upon addition of less than 1 equivalent of alkali the pH rose above 10 and fell gradually during several hours to reach stable values in the region of pH 6.

4-Thiouracil and 2-mercapto-5-amino-1,3,4-thiadiazole were present only in small concentrations in ultrafiltrates of serum to which they had been added. 2-Thiobarbituric acid was mainly filtrable, whereas 5,5-diethylthiobarbituric acid and 2-mercaptoimidazole were somewhat less so. It may be seen that the recoveries from serum with p-toluenesulfonic acid as protein precipitant reflected the degree of binding by serum proteins shown by ultrafiltrations above pH 7; those which were less than 3 per cent ultrafiltrable could not be recovered from serum. 4-Thiouracil was the exception.

Further Observations upon Color Reaction with Modified Grote's Reagent— Thiourea and its N-alkyl derivatives yield a royal blue with Grote's reagent, with maximum absorption near 600 mμ, whereas 2-thiouracil, some of its derivatives, and a number of other structures give a blue-green color, with maximum absorption above 660 mμ. In an attempt to define the structural features which modify the color given by thiourea, parts of the spectra of the colorants due to thiourea, N,N'-tetramethylthiourea, N,N'-ethylenethiourea, 2-mercaptoimidazole, N-acetylthiourea, N,N'-diacetylthiourea, 2-thiouracil and some of its 6-alkyl derivatives, dihydrothiouracil, and 2-thiobarbituric acid were studied. Some of the results are shown in Fig. 1. Alkyl derivatives of thiourea gave spectral absorption curves very similar to that of thiourea, ethylenethiourea (I) giving optical densities almost identical to those given by thiourea (mole for mole), whereas tetramethylthiourea gave somewhat more color. The N-monoacyl derivatives of thiourea, N-acetylthiourea and dihydrothiouracil, gave only slightly

modified curves, whereas with 2-mercaptoimidazole, 2-thiouracil and its 6-alkyl derivatives, 2-thiobarbituric acid, and 2-mercapto-5-amino-1,3,4-thiadiazole the maxima were shifted to above 660 mμ. 2-Mercapto-5-amino-1,3,4-thiadiazole gave a blue-green color with Grote's reagent, despite the absence of the N—C(=S)—N structure.
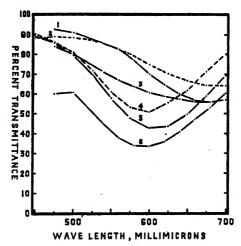
FIG. 1. Spectral transmittance curves of colorants formed with modified Grote's reagent. Curve 1, 0.71 mg. per cent of 2-thiouracil; Curve 2, 3.57 mg. per cent of 2-thiobarbituric acid; Curve 3, 1.16 mg. per cent of 2-mercaptoimidazole; Curve 4, 0.71 mg. per cent of thiourea; Curve 5, 1.43 mg. per cent of dihydro-2-thiouracil; and Curve 6, 14 mg. per cent of N-acetylthiourea.

DISCUSSION

The two compounds ethylene thiourea (I) and 2-mercaptoimidazole (II) illustrate one structural feature which modifies the color reaction. The latter contains a double bond which is in conjugation with the double bond produced by enolization (III). This feature is present also in 2-thiouracil and its 6-substituted derivatives and several other structures which gave a similar color reaction.

The behavior of dihydrothiouracil suggests that the mercapto form of this substance is the one which yields the color reaction, since the formation of a titratable group and a chromogenic structure proceeded at similar slow rates under the same conditions. A greater lability in the desmotropy of thiouracil than of dihydrothiouracil might be anticipated, since a conjugated ring system can result in the former case. The blue color reaction given by thiourea probably does not require the formation of a mercapto group, since N,N'-tetramethylthiourea gave even more color than thiourea. Formation of the mercapto group by desmotropic change would probably be blocked by the methyl groups.

The mercapto form (either in dissociated or undissociated state) appears to be much more strongly bound to proteins than the C=S form. This is suggested by the absence of binding in serum of thiourea (5) which is little dissociated at pH 7 to 8, by the only moderate binding of 2-mercapto-imidazole, which is slightly more dissociated in this region, and by the relation of the binding of thiouracil to pH (3). The behavior of dihydro-thiouracil in passing to a more strongly protein-bound state under conditions in which it was assuming a more titratable structure supports this conclusion.

No direct relationship is to be seen between the thyroid activity of these compounds and their behavior with respect to serum proteins. Among the highly active substances 2-mercaptoimidazole and 5,5-diethylthio-barbituric acid showed the least binding by protein. Of the three compounds showing the highest order of activity, 6-ethyl-, 6-propyl-, and 6-benzylthiouracil, the first two would be recommended if it is accepted that a high affinity for serum proteins is undesirable.

Of the highly active compounds studied analytical procedures for the determination of serum concentrations are provided by these experiments for all but two (6-benzylthiouracil and 2-mercapto-5-amino-1,3,4-thiadi-azole).[3] Satisfactory procedures for deproteinizing serum without losses of these have not been found. Analyses of serum without deproteinization (6) in our hands have not yielded satisfactory analyses with any of these compounds; nor did ultrafiltration of acidified serum permit recovery of benzylthiouracil.

[3] Serum levels of two patients receiving 500 mg. of thiouracil and 6-propylthiouracil respectively reached 1.0 and 1.1 mg. per cent in about 2 hours, and then fell rapidly. Three patients whose thyrotoxicosis was controlled by 6-propylthiouracil showed serum levels from 0.04 to 0.08 mg. per cent. These results indicate that the high activity of propylthiouracil does not arise from its producing much higher serum concentrations than thiouracil. (Unpublished results, Astwood, Christensen, and Cooper.)

SUMMARY

A number of sulfur compounds possessing strong inhibitory effects upon the thyroid gland, and some substances structurally related to them, have been studied with regard to their reaction with Grote's reagent, their analytical determination in serum, and their combination with serum proteins as shown by ultrafiltration. Procedures permitting analytical determination on serum have been described for all but two of the more active compounds studied.

The structural features modifying the color reaction given by thiourea with Grote's reagent have been discussed.

6-Alkyl-substituted 2-thiouracil derivatives were bound by serum proteins to a degree increasing with the size of the substituting group. 2-Mercaptoimidazole and 5,5-diethylthiobarbituric acid, among the highly active substances, were present in serum to a considerable extent in an ultrafiltrable state. Evidence has been presented that the mercapto form of these sulfur compounds is the more strongly protein-bound.

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THE EFFECT OF LECITHINASE ON HUMAN SERUM GLOBULINS

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The protein complex found in human serum and named the X protein by McFarlane (6) has been studied in great detail by Pedersen (8), and assumed to be a complex made up of β-globulin, albumin, and large amounts of lipid. Since the Nagler effect (2, 7) (the release of lipids from solution in serum by the action of the α-toxin of *Clostridia*) is, like the X protein, confined almost entirely to the serum of humans, it seemed of interest to study the relationship between these two phenomena. It has been shown by Macfarlane and Knight (5) that the action of α-toxin is that of a lecithinase. Inorganic phosphate is released by the enzyme from the lecithovitellin of hen's eggs and from pure lecithin. Crook (2) believes the action of α-toxin on human serum to be more complex than this. He found the insoluble material separated from serum after toxin treatment to consist of about one-third protein and two-thirds lipid (fat, cholesterol, phosphatides, etc.). In this paper the action of α-toxin on whole serum, X protein, and various serum fractions has been studied. Although these findings are only a preliminary to a more thorough study, they are reported because the work cannot be continued at this time.

Methods

Serum was prepared from blood drawn from a normal subject (blood group A) in the postabsorptive state. It was allowed to clot at room temperature for about 15 minutes, and then spun in a refrigerated centrifuge for 1 hour. The serum was extremely clear.

The X protein was concentrated as described by Pedersen (8). To 60 cc. of serum, 43 cc. of saturated $MgSO_4$ were added. The solution was spun in sixteen 6 cc. tubes in a concentration ultracentrifuge for 6 hours at 24,000 R.P.M. The effect of $MgSO_4$ is to increase the density of the solvent to such an extent that the X protein separates at the top.

The top 1 cc. from each tube was collected. The combined 16 cc. has been designated as X. The bottom 16 cc. were also collected and combined as a control. This has been designated S-X. The magnesium sulfate was removed by dialysis against cold saline.

The other proteins studied were fractions of normal human plasma prepared by alcohol fractionation (1).

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The lecithinase used was supplied through the courtesy of Dr. Milan A. Logan of the University of Cincinnati, College of Medicine. It was a crude culture filtrate of *Clostridium perfringens* which had been dialyzed against glycerol and contained about 500 mouse LD_{50} per cc.[1] of α-toxin.

This toxin was diluted 1:50 with saline immediately before use. The procedure was adapted from that used by Macfarlane and Knight (5). Usually each flask contained 0.6 cc. of 0.3 M $CaCl_2$, 3.0 cc. of borate buffer, 5.0 cc. of diluted toxin, the protein to be studied, and saline to a final volume of 21.6 cc. In the early experiments the enzyme treatment was carried out in colorimeter tubes and any increase in turbidity measured in the Evelyn photoelectric colorimeter. Later the enzyme action was followed by determinations of phosphate soluble in 10 per cent trichloroacetic acid. 3 cc. of solution were added to 6 cc. of 15 per cent trichloroacetic acid and the precipitate was filtered off after 15 minutes. The phosphorus method of Fiske and Subbarow (3) as modified for the Evelyn colorimeter was used. Samples for inorganic phosphate determinations were taken immediately after adding the toxin. The flasks were stoppered and set in an oven at 37°. After 16 hours another phosphate sample was taken. In the case of the whole serum (S), X, and S-X proteins, the separated lipid was removed by spinning for 2 hours at 24,000 R.P.M. as suggested by Crook (2).

Nitrogen analyses were made by the method of Johnson (4).

Before ultracentrifugal analysis each sample was dialyzed against a buffer containing 0.075 M sodium citrate, 0.0435 M disodium phosphate, and 0.0027 M monosodium phosphate. This buffer was found by Pedersen (8) to give adequate resolution of the albumin and X protein boundaries in the ultracentrifuge. Analyses were made in concentrated solutions, since the X protein complex disappears on dilution. Pictures taken by the diagonal knife-edge method after about 120 minutes at 60,000 R.P.M. were analyzed for X protein, albumin, and globulin. All concentrations were corrected for the sector shape of the cell (9).

Electrophoretic analyses were made in a buffer which was 0.05 M in diethylbarbituric acid and 0.00765 M in sodium citrate and was adjusted to pH 8.6 with sodium hydroxide.

RESULTS AND DISCUSSION

The increase in phosphorus soluble in 10 per cent trichloroacetic acid following lecithinase treatment of a number of serum protein fractions is shown in Table I. Since the albumin showed no significant increase in inorganic phosphate, the values obtained on the other fractions have been recalculated in terms of the total globulin present in each. The results are surprising. Neither the X protein prepared from serum nor that from

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[1] LD_{50} is the amount of toxin that will kill 50 per cent of the mice of the test group.

Fraction II + III showed as great an increase in inorganic phosphorus as the protein mixture from which it was prepared. The greatest amounts of phosphate were released from Fraction III-1 and from the S-X material. The increase in phosphate found with the γ-globulin was probably not significant.

Although the action of lecithinase on the X protein fractions hydrolyzed little phosphate, it did release the lipid. After 2 hours in the oven, the S-X flask still remained clear, while the solution in the X flask had become quite turbid. After 16 hours, a few flocs of fatty material were floating in the S-X sample; the X sample was milky. Nitrogen analyses made before and after the lipid was removed showed that the S-X sample had lost no nitrogen, whereas the X sample had lost 10 per cent.

TABLE I

Action of Lecithinase on Serum Proteins

Protein	Phosphorus freed per gm. total protein	Per cent globulin	Phosphorus freed per gm. globulin	Increase in turbidity	Per cent N₂ removed with lipid
	mg.		*mg.*		
Serum	0.20	36	0.56	++++	5
S-X	0.80	40	2.00	+	0
X from serum	0.04	40	0.10	++++	10
" " Fraction II + III	0.04	100	0.04	++++	
Fraction II + III	0.10	96	0.10	+	
" IV-1	0.05	96	0.05	+	
" III-1	1.00	96	1.00	+++	
Albumin	0.00	1		0	
γ-Globulin	0.01	98	0.01	0	

The results of ultracentrifugal and electrophoretic analyses are shown in Fig. 1. The whole serum contained 23 per cent X (in 4 per cent solution) and 8 per cent β_1-globulin. The X preparation contained 51 per cent X and 20 per cent β_1-globulin. The S-X preparation contained no X, although it had 10 per cent β_1-globulin. After lecithinase treatment the X had disappeared from the serum and the X preparation. The electrophoretic pattern of the treated whole serum (S-L) had changed but little. The X-L pattern, however, showed a loss of 13 per cent of the β_1 boundary. This is consistent with the removal of 10 per cent of the total nitrogen. The percentage increases in the other components seem to be due entirely to the decrease in β_1-globulin. Although it had shown the greatest increase in inorganic phosphate on lecithinase treatment, the S-X fraction gave the same electrophoretic and ultracentrifugal analyses after treatment as before.
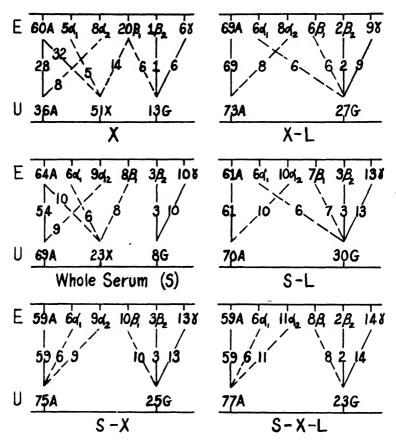
FIG. 1. Electrophoretic (*E*) and ultracentrifugal (*U*) analyses of serum and serum fractions before and after lecithinase treatment. In each diagram the per cent of the various serum proteins found on electrophoretic analysis is indicated along the top horizontal line. The per cent of the total serum protein found in each ultracentrifugal component is marked along the bottom line; *A* indicates the albumin, *X* the *X* protein complex, and *G* the globulins. A connecting line is drawn between each electrophoretic component and the ultracentrifugal component to which it probably corresponds. Solid lines have been drawn for serum albumin and for the β_2- and γ-globulins, since the sedimentation behavior of the isolated proteins is known. Dash lines have been drawn to indicate the probable sedimentation behavior of the other components. Analyses of the *X* protein concentrate (*X*), the whole serum (*S*), and the bottom fraction (*S-X*) are shown in the left-hand diagrams. The right-hand diagrams indicate analyses of the same fractions after lecithinase treatment.

One further effect of lecithinase action must be noted. In all three cases the mobility of the α_1-globulin was reduced from -6.2×10^{-5} sq. cm. per volt per second to -5.6×10^{-5} sq. cm. per volt per second. The boundary separated much more sharply from that of the albumin.

The material (other than the X protein) from which such relatively large amounts of phosphorus were released is unknown. It appears to have a high sedimentation rate, since it is concentrated in the S-X preparation. Some fast moving material was present in the ultracentrifuge diagrams, but in very small amount.

It has been well established by the English workers (2, 5, 7) that the Nagler effect, the release of lipid, is a specific result of lecithinase activity. The increase in inorganic phosphorus found in the S-X preparation cannot be ascribed with certainty to the action of lecithinase, since no control experiments were done. It may be due to the action of other phosphatases present in the serum. This seems unlikely, however, since the S-X preparation was 5 days old when tested, and the Fraction III-1 sample had been stored in the refrigerator for 2 years. The α_1-globulin present in the S-X appeared to sediment with the albumin boundary, which was abnormally large in area.

In every case the α_2-globulin appeared to sediment with the albumin. Pedersen (8) has described the X protein complex as containing β-globulin, albumin, lipid, and carbohydrate. In these preparations the increases in β_1-globulin and in X protein attained in the X preparation are roughly parallel. In the X preparation, however, the complex seems to contain much more albumin than in the whole serum. It also seems probable that some of the α_1-globulin enters into the complex. After lecithinase treatment this fraction of α_1-globulin sediments with the globulins.

SUMMARY

1. The concentration of the X protein complex of human serum by ultracentrifugation has been confirmed.

2. The release of lipid from human serum by the action of lecithinase parallels the breakdown of the X protein complex.

3. The amount of phosphate released from whole serum or from various serum fractions by lecithinase bears no apparent relation to the amount of lipid set free. No increase in inorganic phosphate is obtained when albumin or γ-globulin is used as substrate. Very small increases are found with Fraction II + III, Fraction IV-1, or the X protein concentrates. Large amounts of phosphate are released from Fraction III-1 and from the rapidly sedimenting proteins of whole serum.

4. A further action of the enzyme appears to be a reduction in the mobility of the α_1-globulin.

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THE ACTION OF PHENYLMERCURIC NITRATE

I. EFFECTS ON ENZYME SYSTEMS

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Earlier papers have shown that yeast extracts antagonize the depressing activity of phenylmercuric nitrate on the respiration and growth of yeast (1), on the respiration (2) and growth (3) of skin, and on the growth of molds (4) and certain bacteria (2, 3) (and unpublished). An explanation of these effects requires a knowledge of the mode of action of phenylmercuric nitrate. Inasmuch as yeast extracts have been shown to accelerate catalase (5) and peroxidase (6) activities, to overcome the toxic actions of cyanide and azide but not of urethane and amyl alcohol on respiring systems (7), and to by-pass the cytochrome-cytochrome oxidase oxidation of ascorbic acid (unpublished), it seemed desirable to investigate the effects of phenylmercuric nitrate on several enzyme systems. The present paper deals with the action of phenylmercuric nitrate on the cytochrome oxidase and succinoxidase systems; on the succinic, lactic, and glucose dehydrogenase systems; and on catalase.

EXPERIMENTAL

Methods and Materials—Manometric procedures at 37.5° were used for the oxidase systems. The cytochrome oxidase system, with ascorbic acid as substrate, was measured according to the method of Schneider and Potter (8). The center well of the respirometer flask contained 0.2 ml. of 2 N sodium hydroxide. The main chamber contained 1 ml. of 2.4×10^{-4} M cytochrome c; 0.3 ml. of a freshly prepared solution of 0.114 M ascorbic acid in phosphate buffer, pH 7.4; 0.3 ml. of 4×10^{-3} M aluminum chloride; 0.2 ml. of a cytochrome oxidase-containing tissue extract; 1.0 ml. of phosphate buffer, pH 7.4; and 0.2 ml. of glass-distilled water. In the experimental flasks the water was replaced by basic phenylmercuric nitrate of appropriate concentration.

Schultze's recommendations (9) were followed in setting up the cytochrome oxidase system with hydroquinone as substrate. The center well contained 0.2 ml. of 2 N sodium hydroxide or water. The main chamber contained 1 ml. of 2.4×10^{-4} M cytochrome c; 0.3 ml. of 0.21 M resublimed hydroquinone in phosphate buffer, pH 7.4; 0.3 ml. of 0.18 M semicarbazide hydrochloride neutralized to pH 7.4; 0.2 ml. of oxidase-containing extract;

1.0 ml. of phosphate buffer, pH 7.4; and 0.2 ml. of water which was replaced with phenylmercuric nitrate in the experimental flasks.

The method of Schneider and Potter (8) was followed in setting up the succinoxidase system. The center well contained 0.2 ml. of 2 N sodium hydroxide. The large chamber held 0.4 ml. of 10^{-4} M cytochrome c; 0.3 ml. of 0.4 M sodium succinate, pH 7.4; 0.3 ml. of 4×10^{-3} M calcium chloride; 0.3 ml. of 4×10^{-3} M aluminum chloride; 0.2 ml. of tissue extract; 1.0 ml. of phosphate buffer, pH 7.4; and 0.5 ml. of water, of which 0.4 ml. was replaced by phenylmercuric nitrate in the experimental flasks.

The anaerobic dehydrogenase determinations were carried out by the Thunberg methylene blue method at 37.5° according to the procedure of Green and Dixon (10). The tubes contained 0.9 ml. of a methylene blue solution prepared by mixing 8 ml. of 1:5000 methylene blue with 6 ml. of 0.2 M phosphate buffer at pH 7.2; 0.1 ml. of 0.1 M substrate (succinic acid, dextrose, or lactic acid); and 1.0 ml. of phosphate buffer (replaced by 1.0 ml. of phenylmercuric nitrate in the experimental tubes). The side arm held 1.0 ml. of the enzyme-containing tissue extract which was tipped in after a 15 minute period for evacuation and thermal equilibrium.

Cytochrome c was prepared from beef hearts by the method of Keilin and Hartree (11) with the exception that it was dialyzed against distilled water instead of 1 per cent sodium chloride. Rat heart extract served as a source of cytochrome oxidase as well as of succinic and lactic dehydrogenases. Rat liver was the usual source of glucose dehydrogenase.

For the determination of catalase activity the previously described modification (5) of von Euler and Josephson's procedure (12) was employed. The earlier experiments were performed with a rat liver extract as a source of catalase; the experiments detailed in this paper were made with a crystalline beef liver catalase prepared by the method of Sumner and Dounce (13).

Basic phenylmercuric nitrate (merphenyl nitrate, Hamilton Laboratories) was used. All solutions of chemicals were prepared with glass-distilled water.

Results

Cytochrome Oxidase—After preliminary experiments to determine a suitable concentration of cytochrome oxidase, the effects of phenylmercuric nitrate were studied with ascorbic acid as the substrate. Fig. 1 shows the results of a typical experiment in which phenylmercuric nitrate was used in concentrations from 5×10^{-5} M to 1.7×10^{-5} M. These concentrations were in the range usually employed in the earlier respiration experiments. All depressed the enzyme activity, the magnitude of the depression ranging from 25 to 60 per cent, depending upon the concentration. To be sure that the phenylmercuric nitrate was acting on the enzyme system and not directly on the substrate, for example by forming a difficultly oxidized mercury salt,

experiments were performed to determine the effect of phenylmercuric nitrate on the autoxidation of ascorbic acid in the absence of enzyme. Under these conditions the compound did not affect the oxidation. If allowance were made for the autoxidation, the depression of enzyme activity by phenylmercuric nitrate would be greater than that given above.

Phenylmercuric nitrate also inhibited the oxidation of hydroquinone by the cytochrome oxidase system. Results of a typical experiment, in which
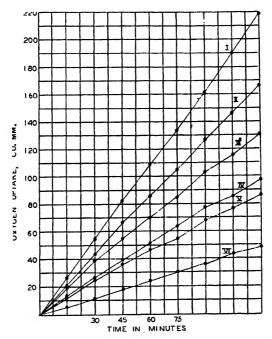
FIG. 1. The effect of phenylmercuric nitrate on cytochrome oxidase oxidation of ascorbic acid. Curve I, cytochrome oxidase control; Curve II, phenylmercuric nitrate, 1.7×10^{-5} M; Curve III, phenylmercuric nitrate, 3.3×10^{-5} M; Curve IV, phenylmercuric nitrate, 4.2×10^{-5} M; Curve V, phenylmercuric nitrate, 5×10^{-5} M; Curve VI, cytochrome c control (oxidase omitted).

2.3×10^{-5} M phenylmercuric nitrate was used, are shown in Fig. 2. Additional experiments showed phenylmercuric nitrate to have no effect on the autoxidation of hydroquinone.

- Spectrophotometric studies (made by Gladys Perisutti) showed that phenylmercuric nitrate did not alter the absorption spectrum of cytochrome c.

Succinoxidase—Phenylmercuric nitrate in concentrations from 2.3×10^{-5} M to 1.25×10^{-5} M gave complete inhibition of the succinoxidase system, as shown by the experiment in Fig. 3.
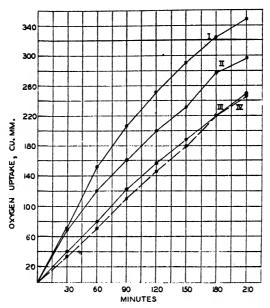
FIG. 2. The effect of phenylmercuric nitrate on cytochrome oxidase oxidation of hydroquinone. Curve I, cytochrome oxidase control; Curve II, phenylmercuric nitrate, 2.3×10^{-5} M; Curve III, cytochrome c control (oxidase omitted); Curve IV, autoxidation of hydroquinone (cytochrome c and oxidase omitted).
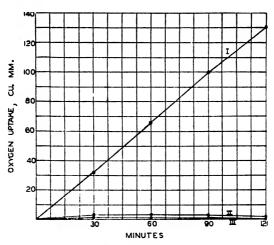
FIG. 3. The effect of phenylmercuric nitrate on succinoxidase activity. Curve I, succinoxidase control; Curve II, cytochrome c control (oxidase and dehydrogenase omitted); Curve III, phenylmercuric nitrate, 1.25×10^{-5} M, 1.7×10^{-5} M, and 2.3×10^{-5} M.

Succinic Dehydrogenase—Table I indicates that phenylmercuric nitrate actively inhibited succinic dehydrogenase, although higher concentrations were required than with the oxidation enzyme preparations.

Lactic Dehydrogenase—Table II demonstrates the inhibition of lactic dehydrogenase by phenylmercuric nitrate.

TABLE I

Effect of Phenylmercuric Nitrate on Succinic Dehydrogenase

Each tube contained 0.9 ml. of methylene blue-buffer mixture and 0.1 ml. of 0.1 M succinic acid. 1 ml. of rat heart extract was tipped in from the side arm.

Phosphate buffer, pH 7.4	Phenylmercuric nitrate	Decolorization time
ml.	M	
1.0		3 min., 2 sec.
1.0		3 " 5 "
	1.1×10^{-3}	1 hr., 12 min.
	1.1×10^{-3}	1 " 9 "
	5.6×10^{-4}	22 min., 5 sec.
	5.6×10^{-4}	22 " 13 "
	2.8×10^{-4}	5 " 31 "
	2.8×10^{-4}	5 " 2 "

TABLE II

Effect of Phenylmercuric Nitrate on Lactic Dehydrogenase

Each tube contained 0.9 ml. of methylene blue-buffer mixture and 0.1 ml. of 0.1 M lactic acid. 1 ml. of rat heart extract was tipped in from the side arm.

Phosphate buffer, pH 7.4	Phenylmercuric nitrate, final concentration 4.5×10^{-4} M	Decolorization time
ml.	*ml.*	
1.0		9 min., 50 sec.
1.0		9 " 43 "
	1.0	17 " 5 "
	1.0	16 " 58 "

Glucose Dehydrogenase—Glucose dehydrogenase activity was depressed by phenylmercuric nitrate but not as readily as succinic or lactic dehydrogenases. For example, in a typical experiment the average control decolorization time for methylene blue was 2 minutes and 29 seconds and 4.5×10^{-4} M phenylmercuric nitrate increased the decolorization time to 3 minutes and 33 seconds.

Catalase—In Table III are reported the depressing effects of phenylmercuric nitrate on crystalline beef liver catalase activity. With 4.5×10^{-5} M phenylmercuric nitrate the depression amounted to approximately 36 per cent within 20 minutes. Similar results were obtained with crude preparations of rat liver.

TABLE III

Effect of Phenylmercuric Nitrate on Catalase

Each flask contained 35 ml. of 30 per cent H_2O_2 diluted 1:500 and standardized,
10 ml. of M/15 phosphate buffer, 1 ml. of standard catalase solution diluted 1:10,000,
and 4 ml. of water which was replaced by phenylmercuric nitrate in the experimental
flasks.

Phenylmercuric nitrate		5 min.	10 min.	15 min.	20 min.
Control	0.1 N $KMnO_4$, *ml.*	4.075	3.475	3.075	2.775
	H_2O_2 decomposed, %	15.12	27.53	35.57	42.15
1.5×10^{-5} M	0.1 N $KMnO_4$, *ml.*	4.375	4.025	3.750	3.400
	H_2O_2 decomposed, %	11.88	20.23	27.01	32.99
3×10^{-5} M	0.1 N $KMnO_4$, *ml.*	4.225	3.425	3.500	3.225
	H_2O_2 decomposed, %	8.76	16.10	21.79	29.09
4.5×10^{-5} M	0.1 N $KMnO_4$, *ml.*	4.500	4.125	3.800	3.500
	H_2O_2 decomposed, %	6.17	13.99	20.75	27.01

DISCUSSION

Phenylmercuric nitrate appears to act as a non-specific depressant of
the enzyme systems studied. This, perhaps, is not unexpected, since
mercury compounds are general protein precipitants. The most obvious
common point of attack of the enzymes is the sulfhydryl group. The action
of inorganic mercury compounds, such as mercuric chloride, has been shown
by Fildes (14) to depend upon interaction with —SH groups. It has been
suggested that organic mercurials may act in a similar manner (15). Or-
ganic mercurials of the type RHgX have frequently been used to inhibit
enzyme activity presumed to be dependent upon —SH (see the reviews
of Hellerman (16)). In Paper II (17), it is shown that the respiratory
depressant action of phenylmercuric nitrate on yeast can be prevented by
such compounds as cysteine and homocysteine.

It is well known that the —SH group is essential for succinic dehydro-
genase activity (18, 19). The depression of this enzyme and of the succin-
oxidase system by phenylmercuric nitrate is in line with this fact. How-
ever, Barron and Singer (20), using chloromercuribenzoic acid, have
reported that the —SH group is not essential for the functioning of cyto-
chrome oxidase and catalase. The depression of the activity of these
enzymes by phenylmercuric nitrate seems to be at variance with the results
obtained with chloromercuribenzoic acid unless the two reagents act in
different manners or unless the concentrations of the depressants employed
were widely different. It would appear that Barron and Singer used
chloromercuribenzoic acid in concentrations of 10^{-3} M. This is considerably

higher than the concentrations of phenylmercuric nitrate (1.5×10^{-5} M to 5×10^{-5} M) which we found to cause definite, although not complete, inhibition of cytochrome oxidase and catalase. It seems, therefore, that the concentration used by Barron and Singer should have produced depression if the action of the two mercurials is similar. The concentrations employed by us were of the same magnitude as the concentrations of chloromercuribenzoic acid (10^{-5} M to 3.3×10^{-5} M) used by Potter and Dubois (19) to produce 30 to 70 per cent inhibition of the succinoxidase system, depending upon experimental conditions. Inasmuch as Paper II shows that the respiratory depressant action of phenylmercuric nitrate on yeast can be prevented by sulfhydryl compounds it would seem that a mode of action involving the sulfhydryl group is a likely possibility.

SUMMARY

Phenylmercuric nitrate depresses the activity of the cytochrome oxidase, succinoxidase, succinic, lactic, and glucose dehydrogenases, and catalase.

These results are discussed in light of a possible action of phenylmercuric nitrate on the sulfhydryl group.

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THE ACTION OF PHENYLMERCURIC NITRATE

II. SULFHYDRYL ANTAGONISM OF RESPIRATORY DEPRESSION CAUSED BY PHENYLMERCURIC NITRATE

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In Paper I (1) it was shown that phenylmercuric nitrate can act as a non-specific depressant for both oxidases and dehydrogenases. Inasmuch as this effect might be interpreted as due to interaction of phenylmercuric nitrate with sulfhydryl groups in the enzymes, we decided to determine whether the depression of yeast respiration by this compound could be prevented by sulfhydryl-containing compounds.

EXPERIMENTAL

Methods and Materials—The respiration of a pure culture of *Saccharomyces cerevisiae* was measured by the previously described procedure (2) in $M/15$ KH_2PO_4 containing 0.02 per cent glucose, pH 4.5, at 30° in Warburg respirometers. In each experiment duplicate flasks were employed for each of the following: yeast control, sulfhydryl-containing (or other) compound, phenylmercuric nitrate, and sulfhydryl (or other) compound plus phenylmercuric nitrate. The control flasks held 0.2 ml. of N KOH in the center well and in the other chamber 3 ml. of phosphate-glucose containing 4 mg., dry weight, of yeast. In the appropriate flasks 1 ml. of the phosphate-glucose was replaced by a solution of the sulfhydryl (or other) compound and 1 ml. was replaced by a solution of phenylmercuric nitrate, which was always tipped from the side arm. All chemicals were dissolved in phosphate-glucose.

$l(+)$-Cysteine hydrochloride, $l(-)$-cystine, dl-homocysteine, and dl-methionine were obtained from General Biochemicals, Inc. Basic phenylmercuric nitrate (merphenyl nitrate) was obtained from the Hamilton Laboratories.

RESULTS AND DISCUSSION

Preliminary experiments revealed that l-cysteine hydrochloride in concentrations from 2×10^{-4} M to 1.3×10^{-2} M depressed the respiration of yeast, the degree of depression being proportional to the concentration up to about 1.6×10^{-3} M. In the range from 3.2×10^{-3} M to 1.3×10^{-2} M the degrees of depression were similar and amounted to 51 to 55 per cent at the end of the 1 hour respiration period. In concentrations of 10^{-4} M,

and lower, cysteine did not depress oxygen consumption significantly and these lower concentrations were chosen for experiments with the phenylmercuric nitrate. Fig. 1 shows the results of a typical experiment. The introduction of 1.5×10^{-5} M phenylmercuric nitrate adversely affected respiration, whereas 10^{-4} M and 6×10^{-5} M cysteine did not. The presence of these concentrations of cysteine materially lessened the depressing effects of phenylmercuric nitrate. Lower concentrations of cysteine were not as effective. It was found that phenylmercuric nitrate would abolish the nitroprusside reaction when present in the ratio of 1 mole to 2 moles of
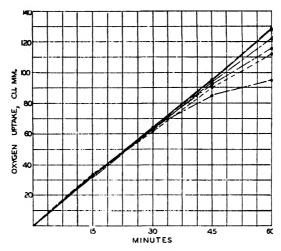
Fig. 1. The effect of cysteine on the depression of yeast respiration by phenylmercuric nitrate. Curve I, yeast control; Curve II, cysteine hydrochloride, 6×10^{-5} M, present from the start; Curve III, cysteine hydrochloride, 10^{-4} M, present from the start; Curve IV, cysteine hydrochloride, 6×10^{-5} M, present from the start; phenylmercuric nitrate, 1.5×10^{-5} M, added at 30 minutes; Curve V, cysteine hydrochloride, 10^{-4} M, present from the start; phenylmercuric nitrate, 1.5×10^{-5} M, added at 30 minutes; Curve VI, phenylmercuric nitrate, 1.5×10^{-5} M, added at 30 minutes.

cysteine. This is the amount of cysteine which should react with all the mercury in the double salt $C_6H_5HgNO_3 \cdot C_6H_5HgOH$, although under certain conditions (3) phenylmercuric nitrate seems to function as the complex ion $(C_6H_5HgOH \cdot C_6H_5Hg)^+$. Somewhat higher than the theoretical amount of cysteine is therefore necessary for effective *in vivo* activity under the conditions of the experiment. Similarly, Fildes (4) found that a greater than theoretical quantity of sulfhydryl compound was essential to antagonize the germicidal action of mercuric chloride on *Escherichia coli*.

dl-Homocysteine behaved similarly to cysteine in preventing the phenylmercuric nitrate depression of yeast respiration.

The presence of *l*-cystine in concentrations of 6×10^{-5} M or 10^{-4} M had no effect upon the respiratory depression caused by the addition of 1.5×10^{-5} M phenylmercuric nitrate, as shown in Fig. 2. Similarly, *dl*-methionine exerted no protective effect against phenylmercuric nitrate.

Unpublished experiments by G. W. Thomas of our laboratories indicate that cysteine antagonizes the germicidal action of phenylmercuric nitrate against *Escherichia coli* and *Eberthella typhosa*. With these organisms, as with the respiration of yeast, higher concentrations of cysteine alone exert a toxic effect.
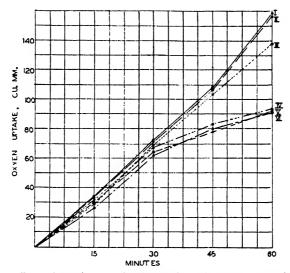
Fig. 2. The effect of cystine on the depression of yeast respiration by phenylmercuric nitrate. Curve I, yeast control; Curve II, cystine, 6×10^{-5} M, present from the start; Curve III, cystine, 10^{-4} M, present from the start; Curve IV, phenylmercuric nitrate, 1.5×10^{-5} M, added at 30 minutes; Curve V, cystine, 6×10^{-5} M, present from the start; phenylmercuric nitrate, 1.5×10^{-5} M, added at 30 minutes; Curve VI, cystine, 10^{-4} M, present from the start; phenylmercuric nitrate added at 30 minutes.

A number of other compounds, including *p*-aminobenzoic acid, adenine, xanthine, and hypoxanthine, which exert an antisulfanilamide effect, are without action upon the respiratory depression of yeast caused by phenylmercuric nitrate (5).

It is evident that the phenylmercuric nitrate depression of yeast respiration can be prevented by sulfhydryl compounds. The inhibition of certain enzyme systems by phenylmercuric nitrate (1) may well be due to interaction with essential —SH groups, although this may not be the only means of causing the observed enzyme inhibition.

SUMMARY

.The depression of yeast respiration by phenylmercuric nitrate can be prevented by cysteine and homocysteine but not by cystine or methionine. It is therefore possible that the previously observed depression of enzyme activity by phenylmercuric nitrate may involve interaction with the sulfhydryl groups of the enzymes.

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FATS OF INSECTS

IV. COMPOSITION OF THE FAT OF MELANOPLUS ATLANIS RILEY

By JOSÉ GIRAL, FRANCISCO GIRAL, AND MARÍA LUISA GIRAL

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One of us, in a former study, observed that sulfur is contained in the oil extracted from a Mexican orthopteran (*Taeniopoda auricornis* Walker, Catantopidae, Acridioidea), found in Córdoba, Vera Cruz. The oil is poisonous to rats (1). The oil was extracted from the male and female separately and the percentage of sulfur was found to differ in the two sexes. It was observed that the toxicity was proportional to the sulfur content, an observation suggesting a linkage of the property of toxicity to a sulfur compound in the oil.

In another study the lipid was extracted from a different orthopteran belonging to the same family (*Melanoplus atlanis* Riley, Catantopidae, Acridioidea), occurring in Actopan, Hidalgo. This lipid was found to have a very high acid value (2), like that occurring in the European cantharid *Lytta vesicatoria* (3). We found sulfur in *Melanoplus atlanis* "fat," though in smaller concentration than in *Taeniopoda auricornis* (2). This paper reports more detailed work on the fat of *Melanoplus atlanis*.

Recent reviews on the chemistry of insect oils can be found in (1) and in ((4) p. 63).

EXPERIMENTAL

Extraction and Fractionation of Total Lipid—About 45,000 male and female insects (2) with a total weight of 9.0 kilos were killed with petroleum ether and dried in the sun to constant weight. 2.2 kilos, or 24.5 per cent of the weight of the fresh material, of dry insect bodies were obtained. The bodies were ground and extracted with petroleum ether (b.p. $<60°$) in a continuous apparatus; the resulting total lipid yielded 18.0 gm. of neutral fat and 53.0 gm. of free fatty acids (d_{35}^{35} 0.96). The neutral fat had a melting point of 39–40° and gave a saponification number of 138.9; it contained 0.0173 per cent of sulfur. No sulfur was found in the acid fraction. A 24 gm. portion of the free fatty acids was fractionated by the lead salt method ((4) p. 370); 3.5 gm. of "solid" fatty acids and 20 gm.

of "liquid" acids were obtained.   Analytical data[1] for the various preparations are given in Table I.

The methyl esters of the "solid" and "liquid" fractions were prepared and submitted to fractional distillation under 2 mm. pressure.   The ana-

TABLE I

Analytical Values for Lipid Fractions from Melanoplus atlanis

	Neutral fat	Free acids		
		Whole	Solid	Liquid
Acid value.....................	0	190.3	195.6	193.8
Iodine "	79.8	114.2	8.3	131.9
Thiocyanogen value...........	44.4	59.7		74.4
Acetyl value..................	4.4	1.0		

TABLE II

Fractions of Methyl Esters of "Solid" and "Liquid" Acids

Fraction No.	Weight	Temperature (2 mm.)	S. e.	Iodine value
	gm.	*°C.*		
S1	0.385	155–163	274.6	4.7
S2	0.820	163–165	292.6	6.1
S3	1.625	Residue	307.5	8.2
L1	2.156	175–180	283.1	89.9
L2	1.720	180–183	292.8	109.4
L3	1.506	183–186	301.5	128.3
L4	1.014	186–215	312.0	140.7
L5	4.952	Residue	326.7	146.2

TABLE III

Approximate Composition of Total Acids

Chain length	Saturated	Unsaturated	Mean unsaturation
	per cent	*per cent*	
C_{16}	7.3	4.1	−2.0 H
C_{18}	12.2	29.9	−2.8 "
C_{20}	2.8	38.4	−3.8 "
C_{22}		5.3	−3.7 "

lytical findings, given in Table II, on calculation by the method of Hilditch ((4) p. 400) yield the approximate composition shown in Table III.

[1] There are some discrepancies between the findings given here and the data reported previously (2).   However, since the specimens used in the earlier work were collected in September, whereas those used in the present work were collected in November, these discrepancies are of little consequence.

In qualitative tests both the neutral fat and the free acid fractions were brominated. In ethyl ether only a faint clouding was obtained, which indicates the presence of small quantities of triethenoid or more highly unsaturated acids. In petroleum ether, abundant precipitates were obtained with both fractions.

Sulfur, Phosphorus, and Nitrogen—Extracts of the neutral fat with boiling water, boiling dilute HCl, hot dilute NaOH, or boiling alcohol failed to give positive reactions with nitroprusside or with sodium plumbite. When the neutral fat was boiled with dilute sodium hydroxide, and the resulting mixture acidified and filtered, the filtrate gave a precipitate with barium chloride, indicating the presence of sulfuric esters in the original material.

No fraction contained phosphorus, nitrogen, or vitamin A (by reaction with antimony trichloride).

The injection of 1 cc. of the neutral fat into a 32 gm. mouse did not produce any symptoms of toxic action.

DISCUSSION

As is the case with the lipids of some other insects (1–3), those of *Melanoplus atlanis* consist mainly of free acids, which constitute 74.7 per cent of the total. The free acids consist of saturated acids, stearic (9.1 per cent), palmitic (6.7 per cent), and arachidic (2.1 per cent), and unsaturated acids, C_{16} (4.4 per cent), C_{18} (31.5 per cent), C_{20} (40.5 per cent), and C_{22} (5.7 per cent). Triethenoid acids of the C_{20}-C_{22} series appear to be present, but linolenic acid is absent. Scarcity of material precluded a demonstration of the complete absence of small amounts of fatty acids below C_{16}. The neutral fat contains sulfur and shows a low saponification value which may be due either to a large proportion of acids of series higher than C_{22} or to the presence of a considerable amount of unsaponifiable material; this is a frequent occurrence among insects (5).

The low acetyl value of both free acids and neutral fat shows the absence of hydroxy acids and also of diglycerides, such as the palmityl diglyceride found in silk worm oil (6).

The predominance of stearic acid over palmitic acid is considered to be exceptional in animal fats. Among the higher animals it is found only in sheep, goat, and puma (American lion)[2] fats. However, it has been found in another insect fat besides that of *Melanoplus atlanis;* namely, that of *Zonabris pustulata* (sub *Mylabris*), an insect from India (7). The fact that stearic acid is the principal saturated fatty acid of certain tropical seeds has no bearing on the composition of the lipids of *Melanoplus atlanis*, which feeds on herbaceous plants. The presence of arachidic acid is to be expected from the great abundance of stearic acid ((4) p. 8).

A greater abundance of unsaturated than of saturated fatty acids appears

[2] Giral, F., *J. Chem. Soc.*, 112 (1945).

to be the rule among Acrididae; it is found not only in *Melanoplus atlanis* but also in the South American locust (8), in *Oxya japonica* (9), and in *Taeniopoda auricornis* (5).

The predominance of unsaturated fatty acids of the C_{20} series found in *Melanoplus atlanis* fat is also found in the lipids of a mollusk, *Mytilus edilus* (10), and of many deep sea fish ((4), pp. 28–50). The fat under study, however, differs from those listed above by a much lower content in palmit-oleic acid.

Hilditch ((4) p. 11) has pointed out that as the zoological scale is as-cended the amount of unsaturated C_{20}-C_{22} acids decreases, whereas the amount of palmitic acid increases. The fat of *Melanoplus atlanis* conforms to this generality but it is doubtful whether all insect fats follow the same rule. On the one hand the finding of heneicosane ($C_{21}H_{44}$) in *Lytta vesicatoria* (3) could be due to decarboxylation of C_{22} fatty acids, which are frequently found, together with higher homologues, in insect waxes. On the other hand in at least one insect fat (6) these acids are known to be absent or present in only small amounts.

Polyethenoid acids are commonly found in insect fats, although not in all species (5). In *Melanoplus atlanis* they belong to the C_{20}-C_{22} series, as is the case in fish fats. It is possible that in numerous reports of the presence in insect fats of linolenic acid (an acid otherwise rarely found in animal fats), the authors may have been dealing with acids belonging to the C_{20} and C_{22} series.

Sulfur is present exclusively in the neutral fraction. It is not present as a phosphosulfatide (11) or as a cyclic sulfate of choline, such as that reported in a mushroom (12), since the fat contains neither phosphorus nor nitrogen. As the sulfur is not extractable by acids or alkalies, it is not present as a sulfonic acid or a urothion type of compound (13). On the other hand, the fact that it is split off from the fat by relatively mild hydrolysis makes it unlikely that it is a simple sulfoxide or sulfone, such as bis-β-hydroxyethyl sulfoxide (14) found in the suprarenals or the dimethyl sulfone that is found in suprarenals (15) and in blood (16) of cattle. Pos-sibly it consists of neutral sulfuric esters of alcohols of high molecular weight; these might be either C_{16}-C_{18} monoalcohols, known to be present in insect waxes, or diglycerides, constituting in the latter case true simple "sulfatides" such as that isolated by Levene from cattle brain (17).

The presence of sulfur in fats appears to be limited to certain insects. We have found it in *Melanoplus atlanis* and in *Taeniopoda auricornis*, which are both Catantopidae, Cyrtacanthacridae. It is absent from *Sphena-rium purpurascens* (18), which is an insect belonging to the Acridioidea but to a different family. We have found no sulfur in a wide variety of vege-table fats (olive oil, sesame oil, coconut oil, and chaulmoogra oil) and of animal fats (butter, hog fat, puma fat, turtle fat).

The one experiment on the toxicity of the neutral fat of *Melanoplus atlanis* is inconclusive. As the supply of material was limited, only one experiment on one mouse could be carried out. Furthermore it was noticed at the time that, after injection, the fat of *Melanoplus atlanis* became solid.

We express our appreciation to Miss María Luisa Cascajares, who furnished us the insects, and to Dr. Cándido Bolívar-Pieltáin for the entomological classification and for his participation in the discussion of these results.

SUMMARY

1. The total lipid of the Mexican orthopteran *Melanoplus atlanis* Riley was fractionated and the components characterized. The outstanding features are the preponderance of stearic acid over palmitic acid and the high proportion of the C_{20} acids in the unsaturated fraction.

2. The fat contains sulfur, as do other insect fats. This is found exclusively in the neutral fat, possibly in the form of neutral sulfuric ester. Phosphorus, nitrogen, and vitamin A are absent.

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FATS OF INSECTS

V. SPHENARIUM PURPURASCENS CHARPENTIER

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The writer has found sulfur to be present (1–4) in the fat of *Taeniopoda auricornis* Walker (1) and in that of *Melanoplus atlanis* Riley (2), two insects from the Catantopidae family (Cyrtacanthacridae) of the Acridioidea (Orthoptera). From preliminary chemical work it appears that this type of sulfur compound occurs in the Catantopidae family, but is absent from other animal or vegetable fats (1–4). The fat from *Taeniopoda auricornis* has been found to be toxic for rats and the toxicity appears to be related to the sulfur content. No conclusive evidence on this particular point has yet been obtained for the fat of *Melanoplus atlanis*.

The present paper reports work on the fat of a third insect, *Sphenarium purpurascens* Charpentier (Pyrgomorphidae, Acridioidea), which is commonly found in the outskirts of Mexico City. This fat has been found to contain no sulfur. It contains a very large proportion of free fatty acids, glycerides being either absent or present in very small amounts. This is a feature common to many insects (2, 4, 5), but not to all (3). There is a considerable amount of unsaponifiable material in the lipids obtained by extraction of the whole dried body of the insects with petroleum ether. The fat is found to be rich in palmitic and stearic acids, the former being more abundant than the latter. Among the unsaturated fatty acids both the C_{18} and C_{20} series are present in considerable proportions, C_{18} predominating. Triethenoid acids of series above C_{18} are also found. The same features are found in the fat of *Melanoplus atlanis*, although in this case stearic acid is more abundant than palmitic acid and the C_{20} unsaturated acids are more abundant than those of the C_{18} series. The essential difference between the fat of *S. purpurascens* and that of *M. atlanis* appears then to be the absence of sulfur in the former. This is all the more interesting in view of the fact that the presence of sulfur is common both to the fat of the *M. atlanis* and to that of the *Taeniopoda auricornis*, while, on the other hand, the fat of the latter differs from that of both *S. purpurascens* and *M. atlanis* by showing no free acids and by the absence of triethenoid acids.

EXPERIMENTAL

The specimens were collected during the month of November, **1943**, in the outskirts of Mexico City (Lomas de Chapultepec), males and females

being mixed, and were killed with petroleum ether.   The mean fresh weight was determined by the counting and weighing of 100, 200, and 500 specimens, and was found to be 0.35 gm. per specimen.   Since the total collection weighed 1210 gm., it represents some' 3450 specimens.   They were dried in the sun to constant weight, and a residue of 390 gm. was obtained (32.2 per cent of the weight of fresh material), which after having been ground was extracted with petroleum ether (b.p. <60°) in a continuous extractor.

The petroleum ether extract was dried with $CaCl_2$, filtered, and the solvent removed by vacuum distillation.   The residue obtained weighed 30.5 gm.   This represents 2.52 per cent of the fresh weight and 7.82 per cent of the dry weight of the starting material.   The residue was a solid mass with an orange-gray color and a characteristic odor reminiscent of

TABLE I

Fractions of Methyl Esters of "Solid" and "Liquid" Acids

Fraction No.	Weight	Temperature (2 mm.)	S. e.	Iodine value
	gm.	*°C.*		
S1	1.342	155–158	266.5	11.1
S2	1.050	158–161	276.3	14.0
S3	0.811	161–169	285.1	17.0
S4	2.289	Residue	302.5	28.0
L1	1.011	155–166	263.5	79.9
L2	1.350	166–170	280.5	95.7
L3	1.330	170–174	290.9	111.1
L4	1.419	174–177	296.3	127.2
L5	1.936	177–185	302.4	148.0
L6	3.908	Residue	317.1	184.8

the odor of fish oils and similar to that of the lipid of *Melanoplus atlanis.* Its chemical constants were as follows: m.p. 26–30°, acid value 148.0, saponification No. 170, ester value 22.0, iodine value 93.8, Hehner's index (total acids) 85.4 per cent, unsaponifiable material 10.9 per cent.   Sulfur and nitrogen were absent.

24 gm. of this preparation were saponified with alcoholic KOH; from the aqueous soap solution 2.61 gm. of unsaponifiable material were extracted with ethyl ether.   The total acids (20.5 gm.) were liberated and separated by the lead salt method[1] into 6.5 gm. of "solid" and 13.0 gm. of "liquid" acids.   These were esterified separately with methyl alcohol and the "solid" and "liquid" methyl esters submitted to fractional distillation under 2 mm. pressure.   The analytical data of the fractions are shown in Table I.   The

[1] The high iodine values found for the "solid" fractions (Table I) are ascribable to the fact that the lead salts were not recrystallized.

approximate composition of the total (free and combined) fatty acids, calculated by the method of Hilditch ((6) p. 400), is shown in Table II.

When a sample of the total "liquid" acids was treated with a solution of bromine in acetic acid, it gave a solid precipitate, insoluble in ethyl ether, which did not melt, but darkened at 200°. This observation suggests that the tri- or polyethenoid acid is not linolenic acid but belongs to the C_{20} series.

The author wishes to express his appreciation to Dr. Cándido Bolívar-Pieltáin for the entomological classification and for his assistance in the discussion of these results.

TABLE II

Approximate Composition of Total Acids

Chain length	Saturated	Unsaturated	Mean unsaturation
	per cent	*per cent*	
C_{14}	2.9		
C_{16}	14.8	9.6	−2.0 H
C_{18}	11.4	35.5	−2.9 "
C_{20}		25.8	−4.7 "

SUMMARY

1. The total lipid of the Mexican orthopteran, *Sphenarium purpurascens* Charpentier, consists mainly of free fatty acids. The approximate composition of the total acids has been determined.

2. The fat of *Sphenarium* (Pyrgomorphidae) does not contain sulfur, a feature in which it differs from that of *Melanoplus atlanis* and *Taeniopoda* (Catantopidae). This leads us to suppose that the sulfur compound is specific for the family Catantopidae.

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THE NATURE OF THE ANTIBACTERIAL AGENT FROM ANEMONE PULSATILLA*

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Extracts of buttercups and *Anemone pulsatilla*[1] exhibit antibacterial action against a wide variety of microorganisms, including Gram-positive, Gram-negative, and acid-fast bacteria (1). The present report deals with the identification of the active principle of *Anemone pulsatilla*.

Steam distillation of the dried and ground plants of *Anemone pulsatilla* resulted in a slightly turbid distillate with globules of a yellow oil floating on the surface. The aqueous fraction when shaken with chloroform yielded, after removal of the solvent *in vacuo*, a pale yellow, irritating oil which completely solidified at room temperature within a few hours. This solid was proved, by mixed melting point, analysis, and color reactions[2] (2), to be identical with anemonin (I). It exhibited practically no antibacterial activity towards *Escherichia coli*, *Candida albicans*, and *Staphylococcus aureus*.[3]

On the other hand, the pale yellow oil was highly active before it solidified, and maintained its activity for many months when held at 5° in 1 per cent aqueous solution. Its identity with protoanemonin (II) (2, 4) was confirmed by comparison with samples synthesized according to the methods of Muskat, Becker, and Lowenstein (4, 5) and of Asahina and Fujita (2). In the pure state it rapidly polymerizes to anemonin and higher polymers.

EXPERIMENTAL

Isolation of Protoanemonin—For the isolation of protoanemonin the procedure of Asahina and Fujita (2) was followed, except that the water-insoluble oil that collected in the steam distillate was removed prior to the extraction with chloroform. This oil, which was devoid of antibacterial

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[1] Obtained from S. B. Penick and Company.

[2] We are indebted to Dr. R. B. Woodward for a sample of anemonin.

[3] Using different methods of testing, Boas and Steude (3) apparently found a high order of activity for anemonin.

activity, was obviously not identical with either protoanemonin or anemonin and was not further investigated.

The protoanemonin was purified by repeated distillation with steam and extraction with chloroform. The solvent was removed under reduced pressure, with a bath temperature of 40–50°. The residue was analyzed within an hour.

Analysis[4]—$C_5H_4O_2$. Calculated, C 62.5, H 4.18; found, C 62.9, H 4.63

Antibacterial Activity—A comparison was made of the antibacterial activity of protoanemonin obtained from *Anemone pulsatilla* with that of the protoanemonin prepared synthetically. For the purposes of this comparison three microorganisms were used, the Oxford strain of *Staphylococcus*, a strain of *Escherichia coli*, and a strain of *Candida (Monilia) albicans*. The tests were carried out by adding various dilutions of the different

TABLE I

Comparison of Antibiotic Activity of Natural and Synthetic Protoanemonin

Protoanemonin tested	Highest dilution active against			
	Staphylococcus (Oxford)	*Escherichia coli*		*Candida albicans*
		Casein hydrolysate broth	Infusion broth	
From *Anemone pulsatilla*..............	60,000	83,000	33,000	100,000
Synthetic (Asahina and Fujita)........	33,000	83,000	33,000	62,000
" (Muskat *et al.*)..............	50,000	83,000	25,000	125,000

preparations to fluid media and seeding with constant amounts of the test organisms. The end titer was taken as that dilution of protoanemonin which completely inhibited growth in a standard period of time. *Staphylococcus aureus* (Oxford strain H) was grown in meat infusion broth of pH 7.5; *E. coli* in two media, a casein hydrolysate medium[5] and a meat infusion broth both of pH 7.5; *C. albicans* was grown in 0.2 per cent glucose infusion broth of the same pH. 4.5 ml. portions of the media containing the antibiotic substance were seeded with 0.5 ml. of a 10^{-3} dilution of a broth culture of the test organism. 24 hour cultures of the *Staphylococcus* and *E. coli* and 48 hour cultures of *C. albicans* were employed to prepare this dilution. The tests were read after incubation at 37° for 18 hours in the case of the *Staphylococcus* and *E. coli* and after incubation at room temperature for 48 hours in the case of *C. albicans*.

The results of the tests are summarized in Table I. It will be seen that

[4] We wish to thank Mr. William Saschek for the analyses.
[5] Identical with the medium described by Fox (6) in which casein hydrolysate is substituted for gelatin hydrolysate.

the antibacterial activity of the protoanemonin from the plants compares well with that of the synthetic products. This furnishes biological evidence that protoanemonin is the substance responsible for the antibacterial activity of *Anemone pulsatilla*.

DISCUSSION

Protoanemonin possesses particular interest, since it appears to be another member of a group of antibacterial agents which includes crepin (7), penicillic acid, and clavacin. These substances are characterized by the presence of a 5-membered unsaturated lactone ring and in addition have a highly reactive double bond system. They are further unique in being active against both Gram-positive and Gram-negative bacteria.

$$\begin{array}{cc}
\text{O} \text{—} \text{C=O} & \\
| \quad\quad | & \\
\text{CH}_2\text{—C—CH=CH} & \\
| \quad\quad | & \\
\text{CH}_2\text{—C—CH=CH} & \\
| \quad\quad | & \\
\text{O} \text{—} \text{C=O} &
\end{array}$$

(I)

$$\begin{array}{c}
\text{HC} \text{=} \text{CH} \\
| \quad\quad | \\
\text{C} \quad \text{C=CH}_2 \\
\diagup\diagdown \\
\text{O} \quad \text{O}
\end{array}$$

(II)

$$\begin{array}{c}
\text{HC} \text{=} \text{CH} \\
| \quad\quad | \\
\text{C} \quad \text{C—CH}_3 \\
\diagup \quad || \\
\text{O} \quad \text{OH} \quad \text{O}
\end{array}$$

(III)

$$\begin{array}{c}
\text{HC} \text{=} \text{CH} \\
| \quad\quad | \\
\text{C} \quad \text{CH=CH}_2 \\
\diagup\diagdown \\
\text{O} \quad \text{OR}
\end{array}$$

(IV)

With this in view certain other lactones were examined with respect to their ability to inhibit the growth of *Escherichia coli*. Angelica lactone (α- or β-), one of the simplest of unsaturated lactones, inhibited growth when diluted 1000 times; anemonin (I), another unsaturated lactone, inhibited growth when diluted 2000 times. Treatment of any of these active lactones with alkali to an extent sufficient to hydrolyze the lactone ring results in loss of antibacterial power.

Butyrolactone exhibited no activity when diluted only 500 times. However, unsaturation *per se* is insufficient to account for the antibacterial activity, as may be demonstrated by a comparison of protoanemonin (II) with such closely related open chain compounds as acetylacrylic acid (III), the hydrolysis product of protoanemonin, and vinylacrylic acid or its ethyl ester (IV, R = H or C_2H_5). Compounds III and IV have no antibacterial activity.

SUMMARY

The antibacterial agent of *Anemone pulsatilla* is shown to be proto-anemonin.

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THE AMINO ACID REQUIREMENTS OF STREPTOCOCCUS FAECALIS AND THE USE OF THIS ORGANISM FOR THE DETERMINATION OF THREONINE IN NATURAL PRODUCTS*

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Many workers (1–6) have successfully used microbiological methods for the assay of several amino acids in natural materials. *Lactobacillus arabinosus* has been used as the test organism in many of these studies. Stokes and Gunness (7) have shown that *Lactobacillus arabinosus* is not satisfactory for the assay of threonine. It was necessary therefore to find another organism which would give a quantitative response to threonine under all conditions. Since Mitchell and Snell (8), Luckey *et al.* (9), and Teply and Elvehjem (10) have used *Streptococcus faecalis*, American Type Culture Collection, No. 8043 (formerly (11) referred to as *S. lactis* R),[1] for the assay of "folic acid" and Snell and Guirard (12) have reported the qualitative amino acid requirements of this organism, the use of *S. faecalis* for the assay of the threonine content of natural materials was investigated.

The amino acid requirements of this organism were studied in detail and suitable techniques were devised for determining the threonine content of various protein materials.

EXPERIMENTAL

The constituents of the medium used in these studies, other than the amino acids, are identical to those reported by Teply and Elvehjem (10). The amino acid composition of the medium used for the preliminary work was identical to that used in earlier work with *Lactobacillus arabinosus* (Schweigert *et al.* (3)), with the exception of glycine and serine which were added at 0.2 and 1.0 mg. levels per tube, respectively. Snell and Guirard (12) showed that these amino acids were also required for *Streptococcus faecalis*. The complete composition of the medium used is given in Table I.

The method of preparing the vitamin and the xanthine solutions was

[1] Niven and associates have shown that *Streptococcus lactis* R is an enterococcus, specifically *Streptococcus faecalis*.

the same as described by Luckey *et al.* (9). At the end of the 72 hour incubation period, the lactic acid was titrated with 0.1 N alkali, thymol blue being used as the indicator. All the other techniques were the same as described by Schweigert *et al.* (3).

The requirement of *Streptococcus faecalis* for each of the amino acids listed in Table I was tested by omitting the appropriate amino acid from

TABLE I

Basal Medium for Assay Studies with Streptococcus faecalis*

Constituent	Amount per tube	Constituent	Amount per tube
	mg.		*mg.*
Glucose...................	200	*l*(+)-Glutamic acid...........	4
Sodium citrate...........	250	*l*-Asparagine..................	4
K₂HPO₄..................	50	*l*(+)-Lysine monohydrochloride........................	2
Salts B		*dl*-Threonine..................	2
MgSO₄·7H₂O...........	2	*dl*-Valine.....................	2
NaCl...................	0.1	*dl*-Isoleucine.................	2
FeSO₄·7H₂O...........	0.1	*dl*-α-Alanine.................	1
MnSO₄·4H₂O...........	0.1	*l*(−)-Cystine.................	2
Adenine..................	0.1	*l*(+)-Leucine................	2
Guanine..................	0.1	*dl*-Methionine...............	1
Uracil...................	0.1	*dl*-Phenylalanine.............	1
Xanthine.................	0.1	*l*(+)-Arginine hydrochloride..	1
	γ	*l*(+)-Histidine monohydrochloride....................	0.5
Thiamine................	2		
Ca pantothenate.........	4	*l*(−)-Tyrosine...............	0.8
Pyridoxine............	12	*l*(−)-Tryptophane...........	1
Riboflavin................	2	Glycine.....................	0.2
Nicotinic acid...........	6	*dl*-Serine....................	1
Biotin....................	0.004		
Folic acid concentrate†...	0.02 (Bₑ)		

* In adapting this medium for the assay of threonine the following changes in amino acid concentration were made in accordance with the results obtained: arginine, 0.5 mg.; serine, 0.5 mg.; and leucine, 1.0 mg. per tube.

† Prepared according to the method of Hutchings *et al.* (13). We are indebted to Mr. Keith McCall for supplying this preparation.

the basal medium. Several levels of each amino acid were then added and from the curves obtained by plotting the number of cc. of alkali used against the amino acid concentration the amount of amino acid required for maximum growth was computed. These results and the titration obtained when none of the amino acid was added are given in Table II. Lack of growth as indicated by low acid production when the amino acid was omitted from the medium was taken as evidence for the essential character

of this amino acid. A summary of several assays indicates that alanine, tyrosine, phenylalanine, and glycine are stimulatory in their action.

TABLE II

Amino Acid Requirement of Streptococcus faecalis

Amino acid	Blank titration, 0.1 N NaOH	Amino acid per tube required for maximum growth	Amino acid	Blank titration, 0.1 N NaOH	Amino acid per tube required for maximum growth
	cc.	mg.		cc.	mg.
l(+)-Glutamic acid.....	1.4	0.50	l(+)-Arginine.........	2.0	0.30
l-Asparagine...........	6.6	0.30	l(+)-Histidine........	1.8	0.10
l(+)-Lysine............	5.2	0.60	l(−)-Tyrosine........	8.4	*
dl-α-Alanine...........	9.2	*	dl-Serine..............	5.1	0.35
dl-Valine..............	2.6	0.50	Glycine...............	7.9	*
l(+)-Leucine...........	3.2	0.25	l(−)-Tryptophane....	3.3	0.075
dl-Isoleucine..........	2.1	0.50	l(−)-Cystine..........	2.7	0.90
dl-Phenylalanine.......	7.6	*	dl-Threonine..........	1.5	0.15
dl-Methionine.........	2.6	0.25	All present...........	11.5	

* Stimulatory.
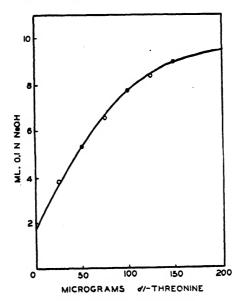
FIG. 1. Typical dl-threonine standard curve

When dl-threonine was used as the standard, it was found to be 50 per cent as active as the naturally occurring isomer[2] (d(−)-threonine). In

[2] We are indebted to Dr. R. T. Major of Merck and Company, Inc., for supplying some of the samples of threonine used in this work.

confirmation of the work of Stokes and Gunness (14) the unnatural isomer[2] ($l(+)$-threonine) was found to be completely inactive. Standard curves were obtained by using dl-threonine over a range of 0 to 250 γ per tube. A typical standard curve is shown in Fig. 1. Samples of muscle and organ tissues, casein, lactalbumin, soy bean oil meal, and ground corn were analyzed for threonine. In all cases the percentage of total nitrogen was determined in duplicate by the Kjeldahl method. The meat samples were also analyzed for moisture and fat.

TABLE III

Proximate Analysis of Animal Tissues

Tissue	Sample No.	Moisture	Fat	Protein
		per cent	*per cent*	*per cent*
Veal	1	74.5	3.7	22.1
	2	74.3	3.9	20.6
	3	74.0	5.3	19.2
	4	74.0	2.5	22.0
Pork	1	54.8	28.5	16.3
	2	51.8	33.8	13.0
	3	55.7	25.4	18.8
	4	49.8	34.0	16.0
Lamb	1	58.2	24.2	16.4
	2	67.1	12.1	18.6
	3	67.7	12.2	18.6
	4	61.4	20.0	16.6
	5	63.1	16.9	17.5
Beef	1	75.2	6.2	17.7
" liver	1	67.4	4.1	19.8
	2	69.5	4.1	20.0
	3	66.8	4.0	23.1
" kidney	1	79.2	3.0	14.5
	2			14.6
" brain	1	79.1	8.8	11.3
	2			10.2
" tongue	1	66.6	16.9	16.0

2.0 N hydrochloric acid was tried as the hydrolytic agent for liberating the threonine from the protein, since this reagent was found to be satisfactory for liberating leucine, valine, and isoleucine from natural materials (3, 4). Maximum liberation of threonine occurred when a 1 gm. sample was autoclaved with 25 cc. of reagent for 2 to 5 hours. An autoclaving time of 5 hours was used for all subsequent assays. The stability of threonine to this procedure of hydrolysis was studied by adding dl-threonine to several different samples of animal tissue prior to acid treatment. In

twelve different trials the recovery ranged from 89 to 115 per cent, with seven of the values falling between 94 and 104 per cent.

TABLE IV

Threonine Content of Natural Foodstuffs

Foodstuff	Sample No.	Threonine	Threonine in protein*	Total N	Threonine N of total N
		per cent	*per cent*	*per cent*	*per cent*
Veal	1	0.99	4.1	3.53	3.3
	2	0.78	3.8	3.30	2.8
	3	0.76	4.0	3.07	2.9
	4	0.96	4.4	3.52	3.2
					3.1 (Average)
Pork	1	0.78	4.8	2.56	3.5
	2	0.62	4.8	2.08	3.5
	3	0.66	3.5	3.01	2.6
	4	0.74	4.6	2.56	3.4
					3.3 (Average)
Lamb	1	0.75	4.5	2.62	3.4
	2	0.80	4.3	2.98	3.2
	3	0.80	4.3	2.98	3.2
	4	0.70	4.2	2.66	3.1
	5	0.68	3.9	2.80	2.9
					3.1 (Average)
Beef	1	0.65	3.7	2.83	2.7
" liver	1	0.91	4.6	2.81	3.5
	2	0.90	4.5	3.20	3.3
	3	1.01	4.4	3.67	3.2
" kidney	1	0.64	4.4	2.34	3.2
	2	0.72	4.9	2.34	3.6
" brain	1	0.53	4.6	1.80	3.4
	2	0.48	4.7	1.63	3.4
" tongue	1	0.76	4.7	2.56	3.5
Ground corn	1	0.40	4.7	1.35	3.5
Soy bean oil meal	1	1.70	3.9	7.02	2.9
Casein*	1	3.18	3.7 (3.8)	13.64	2.7
Lactalbumin*	1	4.75	5.0 (5.2)	15.4	3.7

* All values are calculated to 16 per cent N. The values in parentheses are calculated to 15.6 per cent nitrogen for casein (Chibnall *et al.* (15)) and 15.4 per cent nitrogen for lactalbumin (Jones and Johns (16)).

The proximate analyses of the various animal tissues are given in Table III. In Table IV are summarized the threonine data expressed as per cent of the sample as analyzed, per cent threonine in the protein (calculated to 16 per cent N), and the per cent threonine N of the total N.

DISCUSSION

It is evident from Table II that glutamic acid, asparagine, lysine, valine, leucine, isoleucine, methionine, arginine, histidine, serine, tryptophane, cystine, and threonine give standard responses with *Streptococcus faecalis*. Alanine, tyrosine, phenylalanine, and glycine seem to have only a stimulatory action under the conditions of these experiments. This work suggests that *Streptococcus faecalis* can also be used for the estimation of methionine and histidine.

The qualitative requirements for the amino acids as determined in the present work differ somewhat from those reported by Snell and Guirard (12). They reported that no decrease in the amount of growth resulted when methionine, valine, histidine, and isoleucine were omitted from the basal medium. They also reported that alanine was needed for the growth of *Streptococcus faecalis* but in the present work only a slight increase in growth resulted when levels of alanine up to 0.5 to 2.0 mg. per tube were tested; consequently 1 mg. per tube was used in all subsequent work. Undoubtedly the purity of the amino acids and the different assay techniques employed were largely responsible for the differences noted.

The reliability of *Streptococcus faecalis* for the threonine assay has been shown by excellent recoveries and excellent checks obtained with different levels of the test samples. Autoclaving samples of animal tissues for as long as 18 hours with 2 N HCl did not result in an increase or a decrease in the values obtained. The adequacy of the method of hydrolysis was further established by the fact that no increase in values was obtained after autoclaving for 2 hours with 2 N HCl. In the first half hour as much as 75 per cent of the threonine was liberated. The standard was shown to be reliable, since the naturally occurring isomer gave the same growth response to *Streptococcus faecalis* as twice that amount of *dl*-threonine.

There are no values in the literature for the threonine content of biological material determined by the microbiological method. However, the values obtained in this study compare reasonably well with those obtained by the chemical methods summarized by Block and Bolling (17, 18). The following comparisons can be made when both values are expressed as per cent threonine N of the total N and the microbiological values are given first: casein 2.7 and 2.9, lactalbumin 3.7 and 3.9, soy bean meal 2.8 and 2.9, ground corn 3.5 and 2.6.

It is interesting from a nutritional point of view that most muscle meats contain about 0.7 to 0.8 per cent threonine or approximately 4.0 per cent on a protein basis. When the results are expressed as per cent threonine nitrogen of total nitrogen, the values are remarkably constant for muscle meats. The values for the other tissues tend to be slightly higher but

many more values are needed before any significance can be attached to these small differences. It is clearly evident that lactalbumin contains more threonine than casein, because values of 5.2 and 3.8 are obtained when the calculations are made on the true nitrogen content. The values for the natural products are minimum when expressed as per cent threonine N of the total N, because some of the total N of these samples is non-protein N (Beach *et al.* (19)). It would be valuable to know the exact quantity of protein and non-protein nitrogen in each sample but many difficulties are involved in such determinations. With the improved methods for the estimation of amino acids now becoming available it may soon be possible to determine directly all of the amino acids in biological materials with considerable accuracy. When this can be done, the best procedure may be to calculate the N in each amino acid as per cent of total N and the non-amino acid nitrogen can then be estimated by difference.

SUMMARY

The amino acid requirements of *Streptococcus faecalis* have been investigated. Leucine, threonine, glutamic acid, asparagine, lysine, valine, isoleucine, methionine, arginine, histidine, serine, tryptophane, and cystine were required and alanine, tyrosine, phenylalanine, and glycine were found to be stimulatory.

Streptococcus faecalis has been used for the assay of threonine in natural materials. Suitable recovery experiments and a study of methods of hydrolysis established the validity of the assay.

The following values were obtained: animal tissue protein 4.1 to 4.7, casein 3.7, lactalbumin 5.0, soy bean oil meal 3.9, and whole corn 4.4 per cent threonine in the protein (calculated to 16 per cent N). The values are also expressed for each sample on the basis of the per cent threonine N of the total N.

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DIETARY FACTORS IN THE REGULATION OF LIVER LIPID CONCENTRATION

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The administration of a liver extract (1) or biotin (2) to rats which had previously been fed a low protein diet with no B vitamin supplement for 3 weeks has been observed to result in fatty livers despite the presence of an adequate supply of dietary choline. However, when inositol was also included in the diet, normal liver fat concentrations were found (3). The present paper describes an attempt to determine whether biotin or any other factors present in the liver extract play specific rôles in the regulation of the liver lipids or whether they simply increase the demand for lipotropic factors by stimulating the appetite and growth of previously stunted rats. It was also hoped that the data might explain the need for dietary inositol under such conditions, since choline and methionine suffice to provide normal fat concentrations in the livers of rats fed the usual synthetic rations, and to indicate whether other dietary factors such as tocopherol, vitamin K, and the unsaturated fatty acids also function in this system.

EXPERIMENTAL

The experimental procedure followed, as closely as possible, that used by McHenry and his coworkers (1–5). Male rats of the Vanderbilt strain (6) were grown to a weight of 80 gm. and then housed in individual cages and placed upon the depletion diet (A) for 3 weeks. Less than 6 per cent of the animals failed to survive this period, while the survivors usually weighed between 55 and 70 gm. They were then offered the diets shown in Table I and in addition all animals were given the following supplement by subcutaneous injection daily: thiamine 25 γ, riboflavin 30 γ, calcium pantothenate 100 γ, pyridoxine hydrochloride 20 γ, nicotinamide 50 γ, p-aminobenzoic acid 100 γ. The doses of the other supplements, when given, were choline 30 mg., inositol 30 mg., biotin 5 γ, folic acid 5 γ, α-tocopherol 200 γ, and naphthoquinone acetate 20 γ; and 0.1 cc. of refined corn oil was used as a source of unsaturated fatty acids. Both crystalline folic acid and a potent liver concentrate were used but no differences were observed in their effects. The liver extract was prepared by the procedure of McHenry and Gavin (1) and, when desired, 0.5 cc. was mixed with about 2 gm. of the proper diet and placed on top of the remainder of the

food in each individual feeding cup. This amount of extract provided about 5.5 mg. of choline, less than 2 mg. of inositol, about 2 γ of biotin, and appreciable quantities of folic acid. The latter was assayed against another liver standard and it is not possible to state the actual quantity present. All animals were sacrificed after 7 days, except when stated otherwise, and liver samples taken for analysis. Fatty acids were determined in the usual fashion (8) and cholesterol was estimated by the procedure of Schoenheimer and Sperry (9). The results are summarized in Tables II and III. Each value is the mean for a group of ten rats.

When the low fat diet (B) was fed with only the basal supplement, fatty livers were obtained which were prevented by choline. Inositol appeared to have some lipotropic activity but it was not as marked as that of choline. The administration of liver extract resulted in fatty livers which were somewhat diminished in fat content by additional choline (the extract pro-

TABLE I

Composition of Diets

	Diet A	Diet B	Diet C	Diet D	Diet E
Casein...........................	10	10	10	10	12
Sucrose..........................	84	83.6	63.1	84	71.1
Crisco...........................			20		10
Salts (Hubbell *et al.* (7)).........	4	4	4	4	4
Cellulose.........................	2	2	2	2	2
Sulfasuxidine.....................				1	1
Cystine..........................		0.4	0.4		0.4
Cholesterol......................			0.5		0.5

vided only 5 mg. of choline per day) and more so by inositol, but prevented only by a combination of the two. When biotin was given in addition to the basal supplement, there were again found fatty livers partially resistant to the action of either choline or inositol alone but susceptible to their combined action. However, the biotin effect was not as pronounced as that of the liver extract in stimulating appetite, growth, or liver lipid content. These findings are all essentially in agreement with the findings of McHenry and Gavin (1–4). Since it seemed possible that folic acid might be the substance in liver extract which accounted for the difference between its action and that of biotin, the effects of this compound were studied. When given with the basal supplement, however, it appeared to have no effect on any of the factors which were measured and so it was tried in combination with biotin. The results with this combination were almost identical with those observed when the liver extract was used.

When the high fat diet (Diet C) was employed, it was found that even

without liver extract, biotin, or folic acid, the presence of both choline and inositol was required for the maintenance of normal liver lipid concentrations. It should be noted that Diet C provided 4.5 kilocalories per gm., while Diet B provided 3.7 kilocalories per gm. The effects of the liver extract and of the biotin-folic acid combination were even more pronounced on the high than the low fat diet; so that the administration of choline plus inositol did not provide normal liver lipid concentrations until the 2nd

TABLE II

Lipotropic Activity of Choline and Inositol on Low Fat Diet B Plus Basal Vitamin Supplement

Group No.	Supplement	Weight change	Food intake	Liver fatty acids, mean and standard error	Liver cholesterol
		gm.	*gm. per day*	*per cent wet weight*	*per cent wet weight*
1	None	9.1	6.0	18.7 ± 3.1	0.51
2	Choline	11.7	5.9	5.3 ± 0.4	0.42
3	Inositol	11.2	5.7	16.8 ± 2.9	0.39
4	Liver	22.3	11.6	23.4 ± 3.6	0.93
5	" + choline	24.4	11.1	20.7 ± 1.9	0.82
6	" + inositol	19.7	12.2	8.4 ± 0.6	0.47
7	" + " + choline	23.6	11.9	4.8 ± 0.3	0.36
8	Folic acid	10.3	6.1	20.2 ± 1.7	0.56
9	Biotin	16.8	7.8	19.6 ± 2.4	0.74
10	" + choline	16.0	7.9	8.8 ± 0.5	0.69
11	" + inositol	17.2	7.7	13.6 ± 1.8	0.55
12	" + " + choline	17.6	8.3	4.6 ± 0.3	0.32
13	" + folic acid	21.7	10.8	21.7 ± 3.5	0.88
14	" + " " + choline	19.9	11.7	18.4 ± 2.7	0.80
15	" + " " + inositol	19.0	12.9	17.8 ± 1.9	0.49
16	" + " " + " + choline	21.1	11.9	5.3 ± 0.3	0.31
17	Biotin + folic acid + inositol + tocopherol	19.6	11.8	10.4 ± 0.7	0.41
18	Biotin + folic acid + inositol + corn oil	18.8	12.1	22.0 ± 1.5	0.66
19	Biotin + folic acid + choline 24 days	57.4	8.7	6.1 ± 0.4	0.35

week. Of considerable interest was the observation that on both diets supplementary choline alone was sufficient to obtain normal lipid concentrations even in the presence of liver extract or biotin plus folic acid if the experiment was continued for 24 days. It should also be noted that no differential behavior between cholesterol and liver fat was found. The livers of highest fat content contained the greatest amount of cholesterol and livers of normal fatty acid concentration also were of normal cholesterol content.

Since biotin, folic acid, and inositol are normally available to the rat from synthesis by the intestinal microorganisms, it seemed possible that the effects of these substances might better be studied in animals in which the intestinal synthesis was inhibited by the incorporation of dietary sulfasuxidine (succinylsulfathiazole).   The technique was similar to that above. All animals were depleted for 3 weeks on Diet D which differed from Diet A only in that it contained sulfasuxidine, and then were switched to Diet E which also contained sulfasuxidine and whose fat content was intermediary

TABLE III

Lipotropic Activity of Choline and Inositol on High Fat Diet C Plus Basal Vitamin Supplement

Group No.	Supplements	Weight change	Food intake	Liver fatty acids, mean and standard error	Liver cholesterol
		gm.	*gm. per day*	*per cent wet weight*	*per cent wet weight*
1	None	22.1	6.3	26.1 ± 3.6	0.94
2	Choline	23.3	5.9	11.8 ± 0.8	0.76
3	Inositol	20.8	6.0	17.2 ± 1.9	0.69
4	" + choline	23.8	6.4	7.3 ± 0.6	0.39
5	Liver	40.8	10.4	27.3 ± 4.4	1.37
6	" + choline	36.4	11.1	19.9 ± 3.1	0.90
7	" + inositol	39.1	10.7	14.9 ± 1.7	0.74
8	" + " + choline	42.2	11.4	11.7 ± 0.8	0.63
9	" + " + " 14 days	73.0	9.6	5.8 ± 0.3	0.37
10	Biotin + folic acid	37.9	11.2	25.9 ± 3.7	1.22
11	" + " " + choline	38.4	10.8	18.1 ± 1.6	0.90
12	" + " " + " 14 days	66.8	9.6	11.2 ± 0.6	0.69
13	" + " " + " 24 "	79.3	8.9	6.0 ± 0.5	0.34
14	" + " " + inositol	35.1	10.4	19.7 ± 2.4	0.77
15	" + " " + " + choline	40.2	11.3	10.3 ± 1.8	0.54
16	Biotin + folic acid + inositol + choline 14 days	64.9	10.0	5.1 ± 0.3	0.33

between that of Diets B and C but did contain cystine and cholesterol. The same basal supplement was also given to these animals but without *p*-aminobenzoic acid.   The results are summarized in Table IV.   Each figure represents the mean of a group of ten rats.

The results were essentially in agreement with those found previously. Again choline alone was sufficient to maintain normal liver lipid concentrations (Group 2) unless biotin and folic acid were present (Groups 8 and 15). The effects of biotin alone (Group 10), however, were less notable than in the previous experiments, presumably because of the diminished supply of

folic acid from intraintestinal synthesis, while folic acid alone (Group 11) exerted even less effect than biotin alone. The combination of choline plus inositol always served to regulate the liver lipids unless unsaturated fatty acids were given (Group 9) or tocopherol were lacking (Group 13). However, neither of these effects was very marked and may not be real. No effect ascribable to the presence or absence of vitamin K was noted, although it must be stated that no prothrombin time determinations were performed on the blood of animals in Group 16, and in fact

TABLE IV

Effect of Sulfasuxidine on Liver Fat Concentration

All animals received Diet E + the basal vitamin supplement.

Group No.	Cho-line	Inosi-tol	Bio-tin	Folic acid	Toc-oph-erol	Naph-tho-qui-none	Corn oil	Food intake	Weight change	Liver fatty acids, mean and standard error	No. of rats with hemorrhagic kidneys
								gm. per day	*gm.*	*per cent*	
1	0	0	0	0	0	0	0	5.8	15.2	18.2 ± 2.2	2
2	+	0	0	0	0	0	0	5.9	14.0	4.8 ± 0.3	0
3	0	+	0	0	0	0	0	5.6	15.7	10.1 ± 1.1	2
4	0	+	0	0	0	0	+	5.2	13.7	22.2 ± 3.4	4
5	0	0	+	+	+	+	0	8.8	26.1	27.4 ± 3.2	6
6	+	+	+	+	+	+	0	10.2	37.6	7.1 ± 0.6	0
7	0	+	+	+	+	+	0	9.9	32.4	23.2 ± 2.7	4
8	+	0	+	+	+	+	0	9.6	29.1	16.4 ± 1.8	1
9	+	+	+	+	+	+	+	10.6	35.3	8.2 ± 0.8	0
10	+	0	+	0	+	+	0	7.3	21.5	8.1 ± 1.0	0
11	+	0	0	+	+	+	0	7.0	23.7	7.0 ± 0.3	0
12	+	0	0	0	+	+	0	5.4	14.6	5.0 ± 0.2	0
13	+	+	+	+	0	+	0	10.0	28.1	9.2 ± 0.5	2
14	0	+	+	+	0	+	0	9.3	26.4	28.5 ± 3.6	7
15	+	0	+	+	0	+	0	9.9	30.1	21.0 ± 3.1	4
16	+	+	+	+	+	0	0	10.4	34.0	7.1 ± 0.4	0
17	+	0	+	+	+	+	0	7.9	71.3	8.9 ± 0.7	0 (24 days)

no specific deficiency symptoms of biotin, folic acid, or inositol ever were noted. In contrast to the former results, however, was the finding that rats given choline, folic acid, and biotin still appeared to require dietary inositol after 24 days (Group 17).

The inhibition of the lipotropic action of inositol by unsaturated fatty acids reported by Beveridge and Lucas (10) was confirmed in both series of experiments (Table II, Group 18; Table IV, Group 4). Some suggestive evidence of a synergism between inositol and tocopherol was also obtained in both series. This can be seen in Table II by comparing Groups 15

and 17. In the sulfasuxidine series, when biotin and folic acid were given, in the absence of choline, inositol plus tocopherol (Group 7) was slightly more effective in regulating the liver lipid than was inositol alone (Group 14). Further, even when choline was included with biotin, folic acid, and inositol, slightly fatty livers were found when no tocopherol was given (Group 13), while when choline and tocopherol were not included in the supplement the highest concentrations of liver lipids and the greatest incidence of hemorrhagic kidneys were observed (Group 14). However, there was no evidence that tocopherol exerted any lipotropic influence of itself (Group 5).

DISCUSSION

In the recent work of MacFarland and McHenry (5) it was found that, while inositol alone was sufficient to control liver lipid concentrations when liver extract was administered, the combination of choline and inositol was required when crystalline biotin was given. On this basis the authors sought to distinguish between the biological activity of the liver extract and that of biotin and also between the mechanisms involved in the lipotropic actions of choline and inositol. However, the liver extract used contained about 20 mg. of choline per cc. according to Gavin and McHenry (2) and since the dose used by the former authors was 2.0 cc. per day they supplied approximately 40 mg. of choline per day in the liver extract. In consequence, the addition of further choline to the diet could not have been expected to have any lipotropic action and only inositol was required to secure normal liver lipid concentrations. In our present work, the liver extract provided only 5.5 mg. of choline per day; so that further choline as well as inositol was required to regulate the liver lipids.

While biotin and folic acid, under the specific conditions employed in this work, definitely increased the demand for dietary lipotropic factors, there is no evidence that either of these substances actually plays a specific rôle in lipid metabolism. When liver extract or biotin plus folic acid as well as the basal supplement was included in the diet of rats which had previously existed on a diet seriously deficient in all of these factors, there occurred a much greater increase in food consumption and growth than that seen when only those members of the vitamin B complex which are normally required to be present in the diet of the rat (basal supplement) were given. Under these circumstances then it would seem reasonable to expect an increased demand for lipotropic factors; i.e., choline and inositol. The demand for inositol diminished as the appetite and growth stimulation by the folic acid-biotin combination tapered off. This occurred when the rats had attained a size not much smaller than that of animals of a comparable age on the same basal diet (which is low in protein) supplemented

with the basal vitamin mixture but never previously depleted. At this point also it seems likely that the intestinal flora which normally provides the biotin, folic acid, and inositol for rats on diets devoid of these factors is again established and capable of meeting the requirements for these substances. This is borne out by the finding that when sulfasuxidine, folic acid, and biotin were given there was still a small demand for inositol after 24 days. Such a point of view seems reasonable also when one considers the demand for inositol as well as choline when the high fat diet was fed without any liver extract, biotin, or folic acid. It is consistent also with the previous observations (8, 11–13) that fatty livers due to choline deficiency are not found in animals which are, for any reason, losing weight and that the extent of the accumulation of liver fat in choline deficiency is roughly proportional to the food consumption and growth rate, particularly in young rats. From this standpoint, there is no real justification for the use of the term "biotin fatty liver."

Probably the significance of the present study lies in the suggestion that a diet which is seriously inadequate in the usual accessory food factors may not only result in the deterioration of the host animal but also of the symbiotic microorganisms in the bowel and thus produce a deficiency of the factors ordinarily provided by these organisms for their host. Further, since there is an increased appetite and over-all metabolism during the period of convalescence from any disease in which there is appreciable weight loss, there may then occur an increased demand and consequent relative deficiency in the supply of those vitamins provided by the intestinal organisms, as their synthetic powers may well be limited.

The present work does confirm the lipotropic action of inositol, although it does not suggest any mechanism for this behavior. In contrast to previous suggestions (3, 5) the data do indicate that inositol exerts no selective activity for cholesterol esters rather than the glycerides. The possibilities remain that it functions either by the formation and transport of inositol-containing phospholipids or as a phosphorylating catalyst, perhaps similar to phytic acid, which accelerates the formation of the nitrogenous phospholipids. This possibility is now being investigated. However, it does appear that even in the absence of dietary choline inositol exerts lipotropic activity. The antagonistic action of the highly unsaturated fatty acids was also confirmed.

Of interest is the repeated suggestion that tocopherol may also function in this system. The effects noted, while quite small, were consistent and in keeping with evidence from other sources. Thus Milhorat and Bartels (14) have suggested that a tocopherol-inositol ether may be an active agent in reducing the creatinuria of human muscular dystrophy. Dam and Glavind (15) have found that inositol prevents the exudative diathesis of

vitamin E-deficient chicks, a form of capillary damage which may be similar or analogous to the hemorrhagic kidneys of choline-deficient rats, and that inositol prevents the deposition in the soft tissues of vitamin E-deficient animals (16) of a brown pigment somewhat similar to the ceroid in cirrhotic livers due to choline deficiency (17). In both instances the accumulation of this pigment is dependent upon a source of unsaturated acids, usually a fish liver oil (16, 18, 19). The deposition of such a pigment in the muscle, both smooth and striated, of vitamin E-deficient animals has been a consistent finding (20). While Dam has stated that no tocopherol was found in an alkaline hydrolysate of a lipocaic preparation (16), this problem warrants further investigation, since inositol has been implicated as an active component of such preparations (21) although it cannot of itself entirely account for lipocaic activity (22). Further, we are now determining the effects of inositol and tocopherol on the deposition of ceroid in cirrhotic livers due to choline deficiency.

SUMMARY

The administration of biotin plus a basal B vitamin supplement to rats which had previously been fed a low protein diet with no B vitamins resulted in fatty livers despite the presence of adequate amounts of dietary choline. This was accompanied by a considerable increase in food consumption and growth. The further addition of folic acid accentuated these phenomena, although folic acid without biotin did not produce these effects. Inositol prevented the accumulation of liver fat without affecting either appetite or the growth rate. When a relatively high fat diet plus the basal B vitamin supplement was given to previously depleted rats, inositol was again found necessary to maintain normal liver lipid concentrations even in the absence of supplementary biotin, folic acid, or liver extract, and the effects of biotin plus folic acid were even more pronounced on this diet. However, if the experiments were continued for 24 days, choline alone sufficed to provide normal liver fat concentrations, as the food consumption and growth rate declined after the 1st week with a consequent decrease in the demand for dietary lipotropic factors to a level at which the supply of inositol from synthesis by the intestinal flora was sufficient to meet the inositol requirement. When sulfasuxidine was included in both the deple-

tion ration and the experimental diet, the effects of the biotin-folic acid combination were again manifest but neither alone markedly affected appetite, growth, or liver lipid concentrations. However, the demand for dietary inositol was still apparent even after 24 days, presumably because of inhibition of intraintestinal synthesis. The inhibition of the lipotropic action of inositol by unsaturated fatty acids was confirmed and suggestive evidence was obtained for a synergistic activity of inositol and tocopherol in this system, although tocopherol of itself exerted no apparent lipotropic activity. The significance of these findings is discussed.

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THE EFFECT OF RIBOFLAVIN ANALOGUES UPON THE UTILIZATION OF RIBOFLAVIN AND FLAVIN ADENINE DINUCLEOTIDE BY LACTOBACILLUS CASEI*

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Since new analogues of riboflavin have been synthesized which inhibit the growth of rats and of microorganisms which require riboflavin (1–3), it was decided to test the effect of some of these compounds upon the utilization of riboflavin and flavin adenine dinucleotide (FAD) for growth of *Lactobacillus casei*. Other compounds structurally related to riboflavin were also studied.

It has been shown that isoriboflavin, 5,6-dimethyl-9-(d-1'-ribityl)-isoalloxazine, which inhibits the growth of rats on diets free of or low in riboflavin (3), has less than 0.5 per cent of the activity of riboflavin for *Lactobacillus casei* and has no effect upon acid production by this organism in the presence of maximal amounts of riboflavin (4). The present experiments show that isoriboflavin markedly stimulates acid production by *Lactobacillus casei* in the presence of suboptimal levels of riboflavin or FAD. Snell and Strong (5) observed similar effects of other riboflavin analogues on the growth of lactic acid bacteria.

The ribitylamino compound, 1-ribityl-2-amino-4,5-dimethylbenzene, also has slight riboflavin-like activity and an augmenting effect upon the utilization of riboflavin. However, the addition of alloxan (which can be condensed synthetically with the ribitylamino compound to form riboflavin (6)) permits better utilization of the ribitylamino compound and suggests some synthesis of riboflavin by the bacteria.

The diaminophenazine compound (2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine), synthesized by Woolley, competitively inhibits the utilization of riboflavin for growth of *Lactobacillus casei*, as was reported (2) and has the same effect upon the use of FAD.

Lumiflavin, 6,7,9-trimethylisoalloxazine, has both an inhibitory and a stimulatory action upon the growth of *Lactobacillus casei*. In the presence of relatively large amounts of lumiflavin the growth with riboflavin is

inhibited. As the amount of riboflavin is increased, a zone is reached in which the acid production is increased in the presence of lumiflavin. However, lumiflavin inhibits the utilization of FAD for growth of *Lactobacillus casei* at much lower concentrations than are required for inhibition of riboflavin activity. In this respect the data presented on inhibition by lumiflavin are similar to those reported for thiamine and diphosphothiamine inhibition by pyrithiamine (7).

<div style="text-align:center">EXPERIMENTAL</div>

Methods

The experiments were carried out with *Lactobacillus casei*, and either the alkali-treated peptone medium of Snell and Strong (8) or the hydrolyzed casein medium of Landy and Dicken (9). The biotin content of the latter medium was decreased to one-fifth of that of the original, since it was found to be present in large excess. Growth was determined by pH measurements after 24 hours of incubation at 37° or by titration of the acid produced in 72 hours. For experiments involving titration of acid produced, the glucose concentration of the medium was doubled and the acetate content tripled to provide better growth curves (10). However, for best results with pH values the original concentrations of glucose and acetate were used.

Graded amounts of riboflavin or FAD and other substances to be tested (at pH 6.8) were measured into tubes, diluted to 5 ml., and 5 ml. of medium added. (Some of the compounds studied were sterilized by filtration and added to the cooled tubes after autoclaving, but prior to inoculation.) The tubes were covered with glass caps, autoclaved, and each inoculated with 1 drop of a suspension of *Lactobacillus casei*, prepared by diluting 1 ml. of an actively growing 20 to 24 hour culture with 15 ml. of sterile saline. Unnecessary exposure of all solutions to light was avoided.

Results

Isoriboflavin (5,6-Dimethyl-9-(d-1'-ribityl)-isoalloxazine)—The utilization of isoriboflavin[1] and its effect upon growth with suboptimal amounts of riboflavin and FAD were studied with the peptone and casein media. Table I shows the riboflavin-like activity found on both media with 0 to 0.1 γ of added riboflavin in the presence of 1 to 300 γ of isoriboflavin. The response obtained with isoriboflavin alone is not proportional to the amount present. It is not known whether any part of this activity is due to traces

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[1] A sample of isoriboflavin was generously supplied through the courtesy of Dr. Gladys A. Emerson and her associates at the Merck Institute for Therapeutic Research.

of riboflavin in the isoriboflavin. With the peptone medium the per cent activity of isoriboflavin (compared to riboflavin) decreases from 0.4 to 0.04 as the amount of isoriboflavin is increased from 1 to 300 γ. Conversely, the per cent activity of isoriboflavin added to the casein medium increases from 0.01 to 0.05 as the level of isoriboflavin is increased to 300 γ. The peptone medium supports more growth in the blank tubes than does the casein medium (Fig. 1). This is presumably due to the presence of more riboflavin in the basal peptone medium than in the casein medium.

TABLE I

Stimulation by Isoriboflavin of Utilization of Riboflavin for Growth of Lactobacillus casei

| Growth medium | Isoriboflavin | Riboflavin-like activity found* | | | | |
| | | Riboflavin present | | | | |
		0.0 γ	0.025 γ	0.05 γ	0.075 γ	0.1 γ
	γ	γ	γ	γ	γ	γ
Peptone†	1	0.004	0.05	0.08	0.15	0.18
	10	0.025	0.09	0.14	0.21	0.23
	50	0.06	0.11	0.17	0.25	0.27
	100	0.07	0.14	0.24		0.28
	300	0.11	0.22	0.26		0.31
Casein†	10	0.001	0.12	0.16	0.30	0.40
	50	0.01	0.15	0.25	0.35	0.43
	100	0.06	0.22	0.28	0.37	0.42
	300	0.14	0.27	0.32	0.39	0.41

* Measured by the acid produced in 72 hours. All values are given in terms of microgram per tube (10 ml.).
† See the text.

Since the apparent activity of isoriboflavin is increased in the presence of small amounts of riboflavin, this may partially account for the greater response to the smaller amounts of isoriboflavin on the peptone medium.

In the presence of added riboflavin, however, isoriboflavin stimulates acid production more with the casein medium than with the peptone medium and shows a greater riboflavin-like activity. This may be seen in Table I and Fig. 1. Fig. 1 shows the acid production of *Lactobacillus casei* in the presence of graded amounts of riboflavin on both media, and with 10 γ of isoriboflavin per tube. The same effects of isoriboflavin are obtained when FAD is substituted for riboflavin. The difference in stimulatory action of isoriboflavin on the two media may be due to differences in the protein degradation products in the two media, as is suggested by the following experiments on riboflavin assay of foods and tissues. Isoriboflavin was added to the basal medium (10 γ per tube) in an attempt to increase

the sensitivity of *Lactobacillus casei* to riboflavin (Fig. 1) and to permit more accurate assay of low potency foodstuffs and tissues. The stimulation of isoriboflavin decreased as increasing amounts of food extracts (enzymatic digests or acid hydrolysates) were added, resulting in a "downdrift" of riboflavin values at higher levels of assay. This "downdrift" was greater when the casein medium was employed and was especially marked when foods of high protein content were analyzed.

Ribitylamino Compound (1-Ribitylamino-2-amino-4,5-dimethylbenzene) and Alloxan—The ribitylamino compound[2] has about 0.003 per cent of the activity of riboflavin when incorporated into the medium at levels of 200
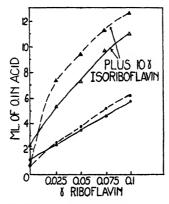
FIG. 1. The effect of isoriboflavin (10 γ per tube) upon the acid produced by *Lactobacillus casei* in the presence of graded amounts of riboflavin. The broken line curves are for the casein medium, the solid lines for the peptone medium. The two lower curves represent acid production with the basal media.

and 400 γ per tube (Table II). In the presence of graded suboptimal amounts of riboflavin, its riboflavin-like activity increases. Alloxan, which can be added to the ribitylamino compound to produce riboflavin synthetically (6), cannot replace riboflavin for growth of *Lactobacillus casei* and has no effect upon its utilization of riboflavin. However, when both the ribitylamino compound and alloxan are added, activities as high as 0.35 per cent of that of riboflavin are obtained (based upon the ribitylamino·compound added). Table II shows the effect of the addition of mixtures of these two compounds either before or after autoclaving. The growth obtained with no added riboflavin and the stimulation in the presence of riboflavin are decreased by autoclaving the ribitylamino compound

[2] The ribitylamino compound was made available by Dr. H. M. Wuest of Hoffmann-La Roche, Inc. This compound was also used in the synthesis of the diaminophenazine analogue of riboflavin.

and alloxan. In the presence of 10 γ of the ribitylamino compound, the molecular equivalent of alloxan (5 γ of the monohydrate) produces the same effect as does a large excess of alloxan (100 γ). The effect of these compounds is the same in the presence of FAD or of riboflavin. Foster (4) has shown that this ribitylamino compound is oxidized by *Pseudomonas riboflavina* 63 per cent as rapidly as is riboflavin. This compound competes with riboflavin for the active centers on the riboflavin-oxidizing enzyme of this organism (4).

Diaminophenazine Compound (2,4-Diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine)—The diaminophenazine compound was synthesized

TABLE II

Stimulation by Ribitylamino Compound and Alloxan of Utilization of Riboflavin for Growth of Lactobacillus casei

Ribitylamino compound	Alloxan mono-hydrate	Riboflavin-like activity found*				
		Riboflavin present				
		0.00 γ	0.025 γ	0.05 γ	0.1 γ	0.15 γ
γ	γ	γ	γ	γ	γ	γ
200		0.009	0.036	0.063	0.12	
400		0.018	0.044	0.09	0.15	
	500	0.00	0.025	0.05	0.10	0.15
25	25	0.05		0.10	0.15	0.20
25†	25†	0.12		0.17		
50	50	0.10		0.15	0.20	
10	5	0.00		0.058	0.13	0.18
10	100	0.00		0.056	0.125	0.18

* The casein medium was used and the riboflavin-like activity was determined by pH measurements after 24 hours.

† In this experiment the ribitylamino compound and the alloxan were sterilized by filtration and added to the test after autoclaving.

by the method of Woolley (2), who has shown that this compound competitively inhibits the utilization of small amounts of riboflavin for growth of *Lactobacillus casei*. The present experiments (Table III) show that riboflavin and FAD are inhibited to the same extent by the diaminophenazine. The inhibition is competitive and is overcome by excess riboflavin or FAD. The inhibition by the diaminophenazine differs from that of lumiflavin (shown below) in that the effects on riboflavin and FAD are the same.

It is interesting to note that the data in Table III show an inhibition of a rather constant amount of riboflavin (about 0.019 γ) by 0.5 mg. of the diaminophenazine in each instance. Similarly, the inhibition of FAD

Sample I by lumiflavin (presented in Table IV) is also constant in each series, and is what would be expected if 25 and 50 γ of lumiflavin counter-

TABLE III

Inhibition by Diaminophenazine Compound of Utilization of Riboflavin and Flavin Adenine Dinucleotide (FAD) for Growth of Lactobacillus casei

Riboflavin or FAD added*	Inhibition of growth in presence of 0.5 mg. diaminophenazine per tube†	
	Riboflavin	FAD
γ	*per cent*	*per cent*
0.0	100‡	100‡
0.02	80	90
0.04	58	55
0.06	28	20
0.08	24	18
0.12	17	17

* The amounts of riboflavin and FAD are given in terms of riboflavin (microgram per tube) for comparison on a molecular basis.

† Diaminophenazine was sterilized by filtration and added after autoclaving. The peptone medium was used and growth measured by pH readings after 24 hours.

‡ The final pH of these cultures was the same as the uninoculated medium. Inoculated blank tubes in the absence of inhibitors show a pH change of 0.2 to 0.3.

TABLE IV

Stimulatory and Inhibitory Effects of Lumiflavin upon Utilization of Riboflavin and Flavin Adenine Dinucleotide (FAD) for Growth of Lactobacillus casei

Riboflavin or FAD added*	Activity in presence of lumiflavin†				
	25 γ lumiflavin		50 γ lumiflavin		
	Riboflavin	FAD Sample I‡	Riboflavin	FAD Sample I‡	FAD Sample II‡
γ	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.025	125	0	20	0	0
0.05	120	50	108	0	0
0.1	120	70	140	50	0
0.15	120	83	120	63	0
0.20		100		75	0

* The amounts of riboflavin and FAD are given in terms of riboflavin (microgram per tube) for comparison on a molecular basis.

† The casein medium was used and growth measured by pH readings after 24 hours.

‡ Sample I of FAD was kindly supplied by Dr. Margaret E. Greig of Vanderbilt University, Sample II by Dr. Oliver H. Lowry of The Public Health Research Institute of the City of New York, Inc.

acted the effect of 0.025 and 0.05 γ of FAD, respectively, at each level at which inhibition is obtained.

Lumiflavin (6,7,9-Trimethylisoalloxazine)—Lumiflavin was prepared from riboflavin by alkaline photolysis according to the method of Warburg and Christian (11). This compound has both an inhibitory and a stimulatory action upon the use of riboflavin or FAD for growth of *Lactobacillus casei* (Table IV). With both the casein and peptone media (24 or 72 hour readings) relatively large ratios of lumiflavin to riboflavin curtail acid production, whereas with smaller ratios (more riboflavin or less lumiflavin), lumiflavin has an augmenting effect. When FAD is supplied to the organism in place of riboflavin, lumiflavin inhibits growth at much lower ratios than are required for riboflavin inhibition (Table IV). This is not due to the inability of *L. casei* to use FAD as well as riboflavin, since in the absence of inhibitors *L. casei* utilizes equivalent amounts of riboflavin, riboflavin phosphate, and FAD equally well for growth and acid production.

The data in Table IV show the effect of lumiflavin upon the utilization of two samples of FAD. These were analyzed for riboflavin microbiologically and were found to contain not more than 2.5 and 10 per cent of FAD, provided all of the riboflavin present was bound as FAD. It is likely that some free riboflavin or riboflavin phosphate was present, especially in Sample I, since this was not inhibited by lumiflavin to the same extent as was Sample II. It was observed that the inhibition by lumiflavin decreased when solutions of FAD were kept in the refrigerator for a few weeks.

The greater inhibition by lumiflavin of the utilization of FAD than of riboflavin by *Lactobacillus casei* is analogous to the increase in inhibition by pyrithiamine when diphosphothiamine replaces thiamine as a growth factor for *Lactobacillus fermenti* (7) and suggests that similar mechanisms may exist for the phosphorylation of these vitamins. Since riboflavin is converted to riboflavin phosphate or to FAD for use in cellular enzymes (12), part of the inhibition of riboflavin activity may be due to blocking of the conversion of riboflavin to one of its conjugated forms. However, lumiflavin inhibits the utilization of FAD more than of free riboflavin. This suggests that lumiflavin competes with both riboflavin and FAD at the place of attachment of the riboflavin moiety to the protein portion of the enzymes, and that in the presence of lumiflavin, riboflavin has a greater affinity for these centers than does FAD. It also indicates that riboflavin can be converted to FAD after it is attached to an enzyme protein.

Hydrolysis of FAD—For assay of riboflavin by microbiological or fluorometric methods, samples are usually hydrolyzed first by dilute acid or digested enzymatically (13, 14). Similar values are obtained by both methods. Abraham (15) has shown that dilute acid hydrolysis splits FAD into riboflavin phosphate and adenylic acid. Experiments with *Lactobacillus casei* were performed to determine the effect of lumiflavin upon the utilization of riboflavin phosphate prepared in this manner, and to ascertain

the extent of digestion of FAD by a mixture of papain and taka-diastase. The results in Table V show that acid-hydrolyzed FAD (riboflavin phosphate) is inhibited less by lumiflavin than is the original FAD. Monophosphothiamine is similarly inhibited less by pyrithiamine than is diphosphothiamine (7). Enzymatic digestion of FAD results in a preparation which responds to lumiflavin-like free riboflavin. Since riboflavin, riboflavin phosphate, and FAD are utilized equally well by *Lactobacillus casei*, and since the fluorescence of riboflavin and riboflavin phosphate is the same,[3] riboflavin values obtained microbiologically or fluorometrically should be the same if complete extraction is obtained by acid or enzyme

TABLE V

Effect of Lumiflavin upon Utilization of Acid-Hydrolyzed and Enzyme-Digested Riboflavin and Flavin Adenine Dinucleotide (FAD) by Lactobacillus casei

Riboflavin or FAD added[a]	Activity in presence of 25 γ of lumiflavin[b]					
	Riboflavin	Acid-hydrolyzed riboflavin	Enzyme-digested riboflavin	FAD Sample II[c]	Acid-hydrolyzed FAD Sample II[c]	Enzyme-digested FAD Sample II[c]
γ	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.05	100	112	110	0	0	110
0.1	120	125	120	0	0	127
0.15	113	120	118	0	34	120
0.20	125	110	120	0	73	118

[a] The amounts of riboflavin and FAD are given in terms of riboflavin (microgram per tube) for comparison on a molecular basis.

[b] The casein medium was used and growth measured by pH readings after 24 hours.

[c] See the corresponding foot-note to Table IV.

digestion. However, FAD provides only about 20 per cent of the fluorescence of riboflavin or of riboflavin phosphate[3] at pH 5, and should be split by one of these methods before fluorometric analysis.

DISCUSSION

Although isoriboflavin competitively inhibits the utilization of riboflavin for the growth of rats (3), it is not oxidized at all by the riboflavin-oxidizing enzyme of *Pseudomonas riboflavina* (4) and it stimulates growth with riboflavin for *Lactobacillus casei*. Analogues of other vitamins have also been shown to differ in their effects upon different animals and bacteria (16, 17). The ribitylamino compound has an effect similar to that of isoriboflavin upon the growth of *L. casei*, but to a much smaller extent. This

[3] Personal communication from Dr. Oliver H. Lowry.

compound, however, is oxidized by *Pseudomonas riboflavina* at 63 per cent of the rate of riboflavin oxidation (4). Upon the addition of alloxan, *L. casei* increases its utilization of the ribitylamino compound from 0.003 per cent of that of riboflavin to 0.35 per cent, suggesting some synthesis of riboflavin from the ribitylamino compound and alloxan.

Banerjee, Dittmer, and du Vigneaud (18) have presented a method for assay of alloxan by condensation with an excess of the ribitylamino compound, and measurement of the riboflavin formed by microbiological or fluorometric techniques. According to the present findings, bacterial growth would be stimulated by the excess ribitylamino compound and by the riboflavin formed. The data of Table II suggest that the preliminary condensation may be omitted and that alloxan may be measured by the growth of *Lactobacillus casei* on a medium containing a known amount of the ribitylamino compound. With both methods adequate correction for the original riboflavin content of the samples would be necessary.

Isoriboflavin and the ribitylamino compound produce the same stimulatory effects when FAD is supplied to the organism in place of riboflavin. The diaminophenazine compound which inhibits the utilization of riboflavin by *Lactobacillus casei* (2) curtails the use of FAD to the same extent. It appears that these three compounds have their main effects upon the use of the riboflavin-containing enzymes and have little or no effect upon the conversion of riboflavin to FAD.

With different ratios of lumiflavin to riboflavin, there are found inhibitory and stimulatory effects of lumiflavin upon the growth of *Lactobacillus casei*. FAD is inhibited by lower concentrations of lumiflavin than is riboflavin. This is analogous to the stronger inhibition by pyridinesulfonic acid of the use of cozymase than of nicotinic acid or amide (19) and to the increased inhibition by pyrithiamine of the utilization of diphosphothiamine than of thiamine (7). All three of these inhibitors seem to compete with the linkage of the respective vitamin or of its coenzyme to the enzyme proteins.

Kuhn and Rudy (20) have postulated that riboflavin phosphate is attached to the enzyme protein at two places; namely, by the 3-imino group in the isoalloxazine ring and through the phosphoric acid group. Diphosphothiamine also appears to be attached to its protein at two points; namely at the 6-amino group of the pyrimidine ring and a phosphoric acid group (21). On the basis of the greater inhibition of the utilization of diphosphothiamine than thiamine by pyrithiamine and by 6-aminopyrimidines, it has been suggested that thiamine is attached to its apoenzyme before being phosphorylated (7). The inhibition of riboflavin and FAD by lumiflavin is similar to the thiamine inhibition and suggests that riboflavin is also attached to its enzyme protein at the 3-imino position before it

is converted to riboflavin phosphate or to FAD. If riboflavin were converted to FAD before being attached to its protein, the utilization of riboflavin should be inhibited by lumiflavin to an equal or greater extent than is found for FAD. Since lumiflavin inhibits the use of FAD more than it does of riboflavin, it appears that the 3-imino group of riboflavin may have a greater affinity for the enzyme proteins than does that of FAD. Although Kuhn and Rudy (20) have shown that riboflavin phosphate is bound to its enzyme more firmly than is free riboflavin, this is due to its two bindings to the protein. Lumiflavin competes only with the linkage of the 3-imino group to the enzyme, and it is the affinity of this group that may differ in the three forms of riboflavin.

In the case of carboxylase, Westenbrink et al. (21) have shown that the naturally occurring form contains diphosphothiamine which is tightly bound to protein, whereas in the reconstructed enzyme (formed by addition of diphosphothiamine to alkaline washed yeast) the linkage is highly dissociable. The intact enzyme is also more active than the reconstituted form (21). Ratner, Nocito, and Green (22) have isolated a flavoprotein enzyme, glycine oxidase, in an undissociated form which is about 4.5 times as active (on the basis of FAD) as the reconstructed enzyme. These authors conclude that the kinetics of combination determine the rate of activity (22). These further similarities between riboflavin and thiamine enzymes make it appear likely that riboflavin, like thiamine, is attached to an enzyme protein *before* it is converted to riboflavin phosphate or to FAD.

SUMMARY

The effects of various analogues of riboflavin upon the utilization of suboptimal amounts of riboflavin and flavin adenine dinucleotide (FAD) by *Lactobacillus casei* have been studied.

Isoriboflavin and 1-ribitylamino-2-amino-4,5-dimethylbenzene possess little riboflavin-like activity by themselves, but are able to stimulate the utilization of riboflavin and FAD by *Lactobacillus casei*. In the presence of alloxan the riboflavin-like activity of the ribitylamino compound is markedly increased.

The diaminophenazine analogue of riboflavin competitively inhibits the utilization of riboflavin and FAD to the same extent.

Lumiflavin can either inhibit or stimulate the use of riboflavin or FAD by *Lactobacillus casei*, depending upon the relative amounts of lumiflavin present. The inhibition by lumiflavin is much greater when FAD is supplied as a growth factor in place of riboflavin. The data suggest that riboflavin may be attached to an apoenzyme before it is converted to riboflavin phosphate or to FAD.

Lactobacillus casei utilizes riboflavin, riboflavin phosphate, and FAD equally well for growth and acid production. FAD is hydrolyzed to riboflavin phosphate by weak acid, and to riboflavin by enzymatic digestion.

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THE FRACTIONATION AND PHOTOMETRIC ESTIMATION OF THE ESTROGENS IN HUMAN PREGNANCY URINE

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In a previous publication (1) we described a liquid chromatogram for separating the trihydroxy estrogen (estriol) from the mono- and dihydroxy estrogens (estrone and α-estradiol). We have modified and extended this technique to accomplish quantitative separation of ternary mixtures of crystalline estrone, α-estradiol, and estriol. Furthermore, we have successfully incorporated the liquid chromatogram in a procedure for the colorimetric estimation of estrone, estradiol, and estriol fractions in human pregnancy urine. Such fractions have been estimated by bioassay techniques (2, 3) but, as the work of Taylor *et al.* (4) has shown, small errors inherent in the fractionation procedures may be enormously magnified by the bioassay.

Bachman and Pettit (5) have adequately discussed the nature of the difficulties encountered in the application of colorimetric methods to the determination of estrogens in human pregnancy urine, in their partial solution to the problem. (Estriol and estrone-estradiol fractions were photometrically determined by them in pregnancy urine containing a minimum of 1000 γ of estriol and 500 γ of estrone-estradiol per liter.) In accord with the Bachman and Pettit concept of the problem we have developed a purification and fractionation procedure which removes most of the chromogenic impurities which might adversely affect reliable photometric determinations with the highly specific Kober reagent (6). This was accomplished by means of the liquid chromatogram.

This report covers (a) a demonstration of the ability of the liquid chromatogram quantitatively to fractionate ternary mixtures of crystalline estrone, α-estradiol, and estriol, and (b) a test of its suitability as an adjuvant to the preparation of estrogenic fractions from human pregnancy urine of sufficient purity for satisfactory colorimetry.

EXPERIMENTAL

Reagents—

Activated alumina, Alorco F-20 "minus 80 mesh" (Aluminum Ore Company, East St. Louis, Illinois), was further screened to 100 to 200 mesh by rapid brushing in standard screen scales (W. S. Tyler Company, Cleve-

land, Ohio) in 100 to 200 gm. batches and stored in tightly stoppered bottles. No batch was kept over 30 days after screening.[1]

Kober's reagent (6) prepared and employed essentially as described by Venning *et al.* (8). Crystalline estrone, α-estradiol, and estriol in the concentration range of 10 to 80 γ were found to obey Beer's law and the following calibration constants were used to calculate the amounts of each estrogen in each test in micrograms: K (estrone) 0.00837; K (α-estradiol) 0.00523; K (estriol) 0.00737. The final Kober color products obtained by testing urine residues from the chromatographic filtrates were further purified by washing with an equal volume of ethyl acetate. This procedure removes impurities without affecting the pink pigment produced by the estrogens. Since the ethyl acetate quickly separates above the aqueous phase, the operation may be carried out in the same colorimeter tube in which the color was developed. Ethyl acetate is somewhat soluble in the final Kober test solution and a number of partition experiments have revealed that the final volume of the ethyl acetate-washed Kober test solution is increased to 17.0 $\pm$ 0.2 ml. Hence, all photometric density measurements on ethyl acetate-washed Kober tests are multiplied by the factor 1.13 in order to utilize the same calibration constants as for untreated tests, for which the final volume is 15.0 ml.

The Bachman phosphoric acid reagent was used essentially as described by Bachman (9).

The Zimmermann reagent (10) was used as described in a previous publication (1).

Normal butyl alcohol (Baker's c.p.) was redistilled under reduced pressure in an all-glass apparatus.

Benzene (Baker's c.p., thiophene-free) was stored over sodium wire and distilled in a dry all-glass apparatus.

Methyl alcohol, absolute (Merck reagent), was stored over activated alumina (Alorco F-20, 80 to 100 mesh) and distilled in a dry all-glass apparatus.

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[1] Standardization studies similar to those reported by Brockmann and Schodder (7) are in progress in this laboratory on a number of adsorbents and will form the subject matter of a subsequent paper. We have found that with ordinary precautions to exclude moisture, surprising uniformity in activated alumina may be maintained. Two 5 pound packages of Alorco F-20, used over a period of 15 months, have shown very slight deterioration. The following visual standardization technique has proved valuable in checking the activity of our preparations: 1.0 ml. of a 0.2 per cent solution of Sudan I (1-benzeneazo-2-naphthol, The Coleman and Bell Company, Norwood, Ohio) in benzene is diluted to 30 ml. with the same solvent and added to a 200 mm. column (13 $\times$ 300 mm. tube). The dye should appear in the filtrate only after washing the column with 45 to 55 ml. of 2 per cent methanol-benzene and should be completely removed from the column after washing with 80 ml.

Ethyl ether, U. S. P., was shaken with a 1 per cent solution of ferrous sulfate in water, washed with water, and distilled. The purified solvent was stored in the refrigerator.

Apparatus—

Chromatographic apparatus. The apparatus previously described (1) was modified to utilize a 13 × 300 mm. instead of a 19 × 200 mm. tube. A cylindrical glass cup of 100 ml. capacity was attached by means of a glass joint to the top of the chromatographic tube.

Urine extractor. The extraction chamber of an unassembled Koch type of extractor (Scientific Glass Apparatus Company, catalogue No. 140, J-1589) was set up to accommodate a motor-driven glass stirrer.

The Evelyn photoelectric colorimeter (11) with the accompanying set of light filters was used for measuring the color intensity of the test solutions. The concentration of estrogen in micrograms was calculated in each test from the formula, $C = L/K$ where $L = 2$ minus the galvanometer reading and where K has been determined by standardization with known quantities of each estrogen.

Procedure

*Preparation of Urine Residue Suitable for Liquid Chromatogram—*The following example will describe the entire procedure. The 24 hour urine specimen was collected in a bottle containing 100 ml. of butyl alcohol as a preservative. Within 24 hours after completion of the collection, the specimen was acidified (to Congo red paper) with hydrochloric acid. It was then extracted four times with 0.125 volume of butyl alcohol by slow stirring in the extraction chamber (150 to 200 R.P.M.). About 5 minutes were allowed for each stirring and a similar interval for separation of the two phases. Each butanol fraction was drawn off and clarified by slow centrifugation. The combined extracts were distilled under a partial vacuum and final traces of solvent removed from the flask by the addition and distillation of 30 ml. of water under similar conditions. The residue was dissolved in 100 ml. of 0.2 N sodium hydroxide and the solution diluted to a 400 ml. volume with water. The aqueous extract was transferred to a 500 ml. wide mouthed Erlenmeyer flask and acidified with 10.0 ml. of concentrated hydrochloric acid. The flask was then covered with an inverted beaker and autoclaved at 15 pounds steam pressure for 3 hours.

The cooled hydrolyzed urine extract was extracted four times with 0.25 volume of ethyl ether. The combined ether extract was washed two times with 0.1 volume of 9 per cent sodium bicarbonate solution and two times with 0.05 volume of water. The ether extract, which should be free from any ether-insoluble material at this point, was then concentrated to a volume of 50 ml. and extracted four times with an equal volume of normal

sodium hydroxide solution. The combined sodium hydroxide extract was backwashed with 0.1 volume of ether, after which it was acidified (to litmus paper) with hydrochloric acid and reextracted three times with 100 ml. volumes of ether. The ether extract was washed two times with 0.1 volume of 9 per cent sodium bicarbonate solution, two times with 20 ml. and once with 10 ml. of 2.5 per cent sodium carbonate solution. The combined sodium carbonate extracts were backwashed with 100 ml. of ether and the backwashing added to the main ether extract. The combined ether extract was then washed with 30 ml. of 9 per cent sodium bicarbonate solution and finally washed two times with 25 ml. of water. The ether was evaporated to dryness and the residue taken up in a measured volume of ethanol from which suitable aliquots were transferred to test-tubes for evaporation. The tubes were stored *in vacuo* over anhydrous calcium chloride until used for preparation of the liquid chromatogram.

Preparation of Liquid Chromatogram—A column of activated alumina of definite length (200 ± 2 mm.) was prepared by filling the assembled apparatus to approximately 20 mm. above the 200 mm. mark and vigorously tapping the side of the tube with a cork grip penholder until the alumina settled to constant height. A negative pressure was then applied. The estrogen residue was treated as described in a previous publication (1) with the following slight modifications. The column was first wet with 20 ml. of pure benzene and the hormone residue (dissolved in 0.4 ml. of absolute methanol and diluted with 20 ml. of pure benzene) added just before the pure benzene had completely entered the column. 50 ml. instead of 75 ml. of benzene were used as a developer. In the distribution experiments 10.0 ml. volumes of the appropriate eluent were successively added to the column and collected in separate flasks. In routine fractionation procedures each eluent (100 ml.) was collected *in toto*. The entire liquid chromatogram operation should not require more than 1 to 2 hours time. After removal of the solvents by distillation, suitable aliquots of the residue were prepared for colorimetry as previously described (1).

Results

Table I summarizes the data from a number of distribution studies on single estrogens in a liquid chromatogram, with various proportions of methanol-benzene mixture as eluents. The results obtained indicate that at least 100 ml. of pure benzene may be used safely without the appearance of a measurable amount of estrogen in the filtrate. Since 2, 5, and 30 per cent methanol-benzene mixtures appeared to be satisfactory eluents respectively for estrone, α-estradiol, and estriol, singly, a number of distribution studies were made on ternary mixtures with these three eluents successively. Fig. 1 shows the distribution of a ternary mixture containing
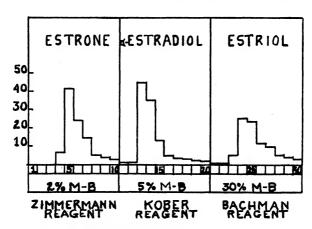
TABLE I

Distribution of Single Estrogens Eluted from Chromatographic Column of Activated Alumina* by Successive 10 Ml. Washings of Various Eluents

Experiment No.	Estrogen, 200 γ	Eluent	Per cent of total estrogen in each washing determined by Kober reagent									
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
1	Estrone	Benzene	0	0	0	0	0	0	0	0	0	0
2	"	2% M.-B.	0	0	0	2	40	30	14	8	4	2
3	"	5% "	0	0	80	8	3	2	0	0	0	0
4	"	10% "	0	80	11	5	0	0	0	0	0	0
1	α-Estradiol	Benzene	0	0	0	0	0	0	0	0	0	0
2	"	2% M.-B.	0	0	0	0	0	0	0	0	0	0
3	"	3% "	0	0	0	0	0	3	13	24	24	18
4	"	5% "	0	0	5	36	30	13	5	3	2	0
1	Estriol	Benzene	0	0	0	0	0	0	0	0	0	0
2	"	20% M.B.	0	0	0	5	8	9	11	15		
3	"	30% "	0	4	20	20	15	10	7	5	3	3
4	"	40% "	5	41	17	11	6	3	2	0	0	0

M.-B. = methanol-benzene.

* Alorco F-20, 100 to 200 mesh, column length 200 mm. in 13 × 300 mm. tube.

FIG. 1. The distribution of a ternary mixture of estrone, α-estradiol, and estriol (1.0 mg. each) in a liquid chromatogram. Each filtrate fraction corresponds to 10 ml. of eluent added. The ordinate gives the percentage recovery of each estrogen and the abscissa, the number of filtrate fractions.

1.0 mg. each of estrone, α-estradiol, and estriol. In these experiments involving mixtures of the estrogens, the Zimmermann test, which is specific among the three estrogens for estrone, and the Bachman phosphoric acid

test, which is specific for estriol, were employed. It can be seen that 100 ml. volumes of each of the three methanol-benzene eluents successively applied to a ternary mixture give adequate chromatographic dispersion of the three estrogens to form the basis for quantitative fractionation.

TABLE II

Separation and Recovery of Known Amounts of Crystalline Estrogens from Ternary Mixtures by Liquid Chromatogram

Experi-ment No.	Amount estrogen added (a)	Color reagent	Total estrogen found (b)			Recovery of added estrogen $\dfrac{(a)}{(b)}$ —
			2 per cent M.-B. (estrone)	5 per cent M.-B. (α-estradiol)	30 per cent M.-B. (estriol)	
	γ		γ	γ	γ	*per cent*
1	200 O.	Zimmermann	200	0	0	100
	50 D.	Kober		55		110
	100 T.	Bachman			75	75
2	100 O.	Zimmermann	105	0	0	105
	200 D.	Kober		180		90
	50 T.	Bachman			38	76
3	50 O.	Zimmermann	50	0	0	100
	100 D.	Kober		100		100
	200 T.	Bachman			160	80
4	No O.	Zimmermann	0	0	0	
	No D.	Kober	0	0		
	1000 T.	Bachman			750	75
5	200 O.	Zimmermann	208	0	0	104
	100 D.	Kober		110		110
	300 T.	Bachman			220	74
					25*	8
6	500 O.	Zimmermann	540	0	0	108
	500 D.	Kober		520		104
	2000 T.	Bachman			1600	80
					125*	6
					61*	4

O. = estrone; D. = α-estradiol; T. = estriol.   M.-B. = methanol-benzene.

* 50 ml. of additional 30 per cent methanol-benzene eluent were added.

Determination of Estrogen Content of Known Solutions—Table II presents data from a number of experiments performed to test the quantitative features of the fractionation process. In these experiments, each eluent was collected *in toto* and tested for estrogen content by means of the three color reagents. A 10 ml. washing of the column with pure benzene was routinely interspersed between the 2 and 5 per cent methanol-benzene eluents and collected with the 2 per cent methanol-benzene filtrate. There is maximal recovery of estrone and α-estradiol in the appropriate fractions and there is consistent recovery, to the extent of 70 and 80 per cent of the

estriol, in the first 100 ml. of the 30 per cent methanol-benzene filtrate. A number of experiments similar to Experiments 4 to 6 in Table II have demonstrated that the low recovery of estriol is in no way attributable to loss of estriol in the preceding chromatographic fractions, but rather to the diffuse nature of the trailing boundary common to many chromatographic zones. Higher concentrations of methanol in benzene or further washing with 30 per cent methanol-benzene causes contamination of the eluate with impurities which impair the use of color reagents, especially when urine residues are involved.

Recovery of Estrogens Added to Essentially Estrogen-Free Urine Extract— Table III summarizes the data from a series of recovery tests in which known amounts of the three estrogens were added, at various points in the purification process, to extract of pooled urine from a castrated female. The amounts added were of an order anticipated to simulate pregnancy urine. Experiments 1 and 2 in Table III indicate that the reaction of the Kober reagent proceeds independently with estrogens and with urinary impurities present in the filtrate residues. The recovery of estrone and α-estradiol added to the urine extract just previous to application of the liquid chromatogram is maximal and recovery of estriol is in the range of 75 to 80 per cent, which would be expected from results with simple benzene solutions (Table II). Experiments 3 to 5 in Table III show that known amounts of estrone and α-estradiol added immediately after hydrolysis to the butyl alcohol extract of castrated female urine may be carried through the entire purification and fractionation procedure and recovered in satisfactory yields. The low recoveries (57 to 69 per cent) in this series of added amounts of estriol were materially improved in subsequent studies with pregnancy urine by inclusion of an ether backwashing of the aqueous sodium carbonate washings.[2]

Recovery of Estrogens Added to Pooled Pregnancy Urine Extracts Immediately after Hydrolysis—Table IV contains a few of a number of experiments which were carried out to test the adequacy of our extraction and purification procedures when applied to pooled pregnancy urine extracts subsequent to hydrolysis. These and other similar studies have demonstrated that estrone and α-estradiol can be recovered in the appropriate filtrate fractions in the range of 80 to 100 per cent and estriol in the range of 75 to 83 per cent.

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[2] Partition studies of estriol between equal volumes of ethyl ether and 2.5 per cent sodium carbonate solution in our laboratory have averaged 60 per cent recovery of the estriol in the organic solvent phase. This is significantly lower than the Bachman and Pettit (5) recovery of 80 per cent with the 9.0 per cent sodium carbonate solution and more nearly in agreement with Mather's (12) value of 65 per cent with 0.3 M sodium carbonate.

Measurement of Estrogens Excreted by Normal Subjects in Early to Mid-Pregnancy—Preliminary observations on the estimation of estrone, estradiol, and estriol fractions in 24 hour urine specimens are presented in Table V. The ratio of L-520 mμ/L-420 mμ for each Kober color product

TABLE III

Recovery of Crystalline Estrogens Added to Extract of Pooled Urine of Human Female Castrate, Subsequent to Hydrolysis

The results are expressed as micrograms per unit aliquot (800 ml. of urine).

Experiment No.	Point at which estrogen is added	Estrogen added	Filtrate fraction No. tested*	Urine equivalent used for colorimetry	Total estrogen content by Kober reagent	$\dfrac{L\text{-}520}{L\text{-}420}$	Per cent recovery of added estrogen†
		γ		*ml.*	γ		
1		None	1	400	15‡	0.59	
			2	400	19‡	0.65	
			3	400	30‡	0.62	
2	Residue just before colorimetry	50 O.	1	400	70	2.6	110
		50 D.	2	400	65	2.1	92
		50 T.	3	400	77	2.4	94
3	Residue just before adsorption	500 O.	1	80	540	9.2	105
		250 D.	2	200	268	5.9	100
		1000 T.	3	40	840	8.9	81
4	Aliquot just after hydrolysis	500 O.	1	80	470	8.2	91
		250 D.	2	200	244	4.5	90
		1000 T.	3	40	620	7.5	59
5	" "	500 O.	1	80	490	8.5	95
		200 D.	2	200	190	3.0	86
		1000 T.	3	100	715	10.5	69
6	" "	500 O.	1	80	500	8.0	97
		500 D.	2	200	490	6.5	94
		1000 T.	3	40	600	7.8	57

O. = estrone; D. = α-estradiol; T. = estriol.

* Fraction 1 = 2 per cent methanol-benzene; Fraction 2 = 5 per cent methanol-benzene; Fraction 3 = 30 per cent methanol-benzene.

† Calculated by subtracting the contribution of atypical Kober color products in Experiment 1 and dividing by the amount of each estrogen added.

‡ By direct determination at 520 mμ with the Kober reagent. These atypical color products do not give true estrogen content but must be included in calculation of estrogen recovery values for Experiments 2 to 6.

has been included in Table V in order to indicate the spectral purity of the test solutions. Venning *et al.* (8) have found, and Bachman and Pettit (5) have confirmed their finding, that the L-520 mμ/L-420 mμ ratio of the Kober products obtained with urinary impurities is approximately 0.5. Experiments on a large number of urine specimens from castrated and preadolescent females have given similar results by our method. Bachman

TABLE IV

Recovery of Known Amounts of Crystalline Estrone, α-Estradiol, and Estriol Added to Aliquots of Pooled Pregnancy Urine Extracts Immediately after Hydrolysis

The results are expressed as micrograms per unit aliquot.

Experiment No.	Amount of estrogen added	Filtrate fraction No.*	Total estrogen found by Kober reagent†	Per cent recovery of added estrogen
	γ		γ	
1	None	1	143	
		2	110	
		3	1380	
2	500 D.	1	140	
		2	592	96
		3	1200	
3	500 O.	1	560	83
	1800 T.	2	136	
		3	2800	76
4	500 O.	1	577	87
	400 D.	2	480	92
	940 T.	3	2080	75

O. = estrone; D. = α-estradiol; T. = estriol.

* Fraction 1 = 2 per cent methanol-benzene; Fraction 2 = 5 per cent methanol-benzene; Fraction 3 = 30 per cent methanol-benzene.

† The final Kober color product was washed with an equal volume of ethyl acetate.

TABLE V

Estrogen Content of Human Early Pregnancy Urine

Experiment No.	Time after last catamenia	$\dfrac{L\text{-}520\ m\mu}{L\text{-}420\ m\mu}$ for Kober color products			Estrogen content		
		Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol
	wks.				γ *per 24 hrs.*	γ *per 24 hrs.*	γ *per 24 hrs.*
1	4	1.2	0.9	1.2	86	108	90
2	8	2.7	1.2	3.4	124	61	142
3	8	4.1	2.2	2.3	209	127	215
4	10	5.1	1.7	3.6	256	134	266
5	18	9.2	1.9	11.8	740	206	1480
6	20	6.4	1.5	11.0	800	270	3390
7	22	5.9	3.4	10.8	875	484	3710
8	23	7.6	3.6	10.8	965	520	4210
9	24	5.5	3.3	9.7	1024	520	4300
10	25	6.7	3.7	12.0	848	500	4400
11	26	7.0	4.4	14.0	1000	570	4400
12	28	6.1	3.9	12.0	1460	900	7000

and Pettit (5) have indicated that, with ratios of $L\text{-}520\ m\mu/L\text{-}420\ m\mu$ as low as 3.0 for contaminated Kober color products, the error in determining the estrogen content directly from the extinction at 520 $m\mu$ should not

,exceed 10 per cent. If we accept this ratio as the lower limit for satisfactory colorimetry, our data in Table V, together with those of unreported experiments, appear to indicate that satisfactory purification of the estrone and estriol fractions may be anticipated from pregnancy urine collected after the 10th week following the last catamenia. The estradiol fraction consistently gives the lowest L-520 mμ/L-420 mμ ratios and usually does not rise above 3.0 until after the 24th week following the last catamenia. Satisfactory Kober color products for all three fractions may be expected from pregnancy urine collected from the 24th week to term, in which period the ratio of estrogens to impurities is most favorable for good colorimetry.

DISCUSSION

Venning *et al.* (8) have shown that normal butyl alcohol satisfactorily extracts both conjugated and unconjugated forms of the female sex hormones from human pregnancy urine, and that the aqueous extract of the butyl alcohol provides a convenient extract for carrying out the hydrolysis of the conjugated forms. Because of its greater freedom from pigmented non-estrogenic material, we have adopted the butyl alcohol extraction and hydrolysis technique essentially as performed by them. It is desirable to fractionate the hydrolysate into neutral and acidic fractions by distribution between ether and aqueous alkali before employment of the liquid chromatogram, since neutral (androgenic) 17-ketosteroids and other neutral ether-soluble substances might conceivably pass into the liquid chromatogram fractions with the estrogens and interfere with development of color with the Kober reagent. Crystalline androsterone, dehydroisoandrosterone, and pregnanediol by actual test appeared in the 2 per cent methanol-benzene filtrate.

No attempt has been made in this study to check the colorimetrically determined hormone titers against bioassay determinations. These colorimetric values may well represent summations of space isomers as yet undetected in human pregnancy urine. For example, the estradiol fraction might conceivably contain both α and β forms, although according to the work of Pearlman and Pearlman (13) this seems unlikely.

In addition to fractionating the mixture the liquid chromatogram accomplishes a measure of purification. Our urines gave a very slight observable residue in the initial filtrate (estrogenically inactive when tested with the spayed rat). There usually is a very weak yellow band which acts as a pace-maker for the estrone fraction. For the present, this has been allowed to pass into the estrone fraction and its presence poses no serious obstacle to the use of the Kober reagent. Fortunately, the major portion of non-estrogenic contaminants of pregnancy urine residues at the conclusion of

the liquid chromatogram is still fixed at the very top of the chromatographic column.

Within the limitations discussed in connection with Table V, the procedure which we have described is believed to offer a definite contribution to the fractionation and quantitative determination of the estrogens known to be present in human pregnancy urine.

SUMMARY

A liquid chromatogram has been described which successfully fractionates ternary mixtures of crystalline estrone, α-estradiol, and estriol. The quantitative features of the technique have been investigated for various ternary mixtures. Estrone and α-estradiol are recovered quantitatively in the 2 and 5 per cent methanol-benzene filtrates respectively, and estriol in the 30 per cent methanol-benzene filtrate to the extent of 75 to 85 per cent. The unrecovered estriol is not lost to the other two fractions, but escapes elution under the conditions employed. The liquid chromatogram has been incorporated in a purification procedure for the estrogens in human pregnancy urine. This procedure yields estrone, estradiol, and estriol fractions which respond satisfactorily to the highly specific Kober reagent.

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THE INFLUENCE OF INGESTED CHOLINE UPON CHOLINE-CONTAINING AND NON-CHOLINE-CONTAINING PHOSPHOLIPIDS OF THE LIVER AS MEASURED BY RADIOACTIVE PHOSPHORUS*

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In an earlier report it was shown that the oral administration of 300 mg. of choline chloride per kilo of body weight increases the rate of change in the specific activity of plasma phospholipid phosphorus during the early intervals after the administration of radioactive inorganic phosphorus (1). Since plasma phospholipids are synthesized mainly in the liver (2), it was suggested that the primary action of choline is on the liver and that the increased phospholipid activity of plasma resulting from ingested choline merely reflects such accelerated activity in the liver.

Since the liver contains C. C.[1] as well as N. C. C. phospholipids, it became of interest to determine whether one or both of these types of phospholipids are influenced by ingestion of choline. This has been done in the present investigation. Measurements of specific activity recorded here necessitated the separation of C. C. from N. C. C. phospholipids of the liver. This was made possible by the use of an adsorption procedure recently developed in this laboratory (3).

EXPERIMENTAL

The dogs used in this study were fed a high fat-low protein diet composed of 38 per cent lard, 8 per cent crude casein, 44 per cent sucrose, 2 per cent sardilene,[2] 3 per cent brewers' yeast, and 5 per cent Cowgill's salt mixture (4). 10 gm. of this mixture per kilo of body weight were fed once daily to each dog for 12 to 16 days before the experiments described below were undertaken.

In each experiment two dogs were used; both received radioactive phosphorus but only one received choline. Each dog was injected intravenously with 10 cc. of an isotonic solution of Na_2HPO_4 containing approximately 0.5 millicurie of P^{32}. The choline was administered by stomach tube; each

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[1] The terms choline-containing and non-choline-containing have been abbreviated to C. C. and N. C. C.

[2] Each cc. of sardilene contained not less than 100 A. O. A. C. chick units of vitamin D and 600 U. S. P. units of vitamin A.

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dog described below as choline-treated received 300 mg. of choline chloride per kilo in 50 cc. of water exactly 30 minutes before the radiophosphorus was injected.   At the time of these administrations all dogs were in the postabsorptive state, approximately 18 hours having elapsed since the ingestion of their last meal.   Food was withheld after the administration of the radiophosphorus.

Samples of blood and liver were removed from each dog at intervals of 6, 12, 18, and 24 hours after the administration of radiophosphorus.   At the 6 hour interval the dogs were anesthetized lightly by an intravenous injection of pentobarbital.   Blood was then quickly removed from the femoral artery.   Heparin was used as anticoagulant.   The abdominal cavity was then opened and the liver exposed.   With the aid of a towel a lobe of the liver was grasped in both hands and a piece of the periphery of this lobe (approximately 3 to 4 gm.) removed.   Bleeding was retarded by means of pressure and completely stopped by momentary applications of a hot iron to the cut surface.   The abdominal cavity was then closed by sutures and the dog kept warm until the next sample was taken.   All liver samples were taken as described above, each being removed from a separate lobe.

Phospholipid P^{31} and phospholipid P^{32} of plasma were measured by procedures previously described (1).

Liver samples were rapidly weighed, transferred to flasks containing 95 per cent ethyl alcohol, and immediately extracted.   The preparation of petroleum ether extracts of liver lipids has been described elsewhere (3).   Choline, phospholipid P^{31}, and phospholipid P^{32} were determined on aliquots of this petroleum ether extract (5, 6).   On another aliquot, the C. C. and the N. C. C. phospholipid were separated after the manner of Taurog et al. (3), and the methanol eluate obtained analyzed for choline, phospholipid P^{31}, and phospholipid P^{32}.   This separation is based on the finding that all phospholipids in a petroleum ether solution are adsorbed on magnesium oxide and that subsequent treatment with methanol elutes only the C. C. phospholipids.

The molal ratios of choline to phospholipid P^{31} for both petroleum ether extracts and methanol eluates are recorded in Table I.   The ratios for the petroleum ether extract fluctuated between 0.50 and 0.66; the values obtained were in fairly good agreement among the six dogs studied.   This means that approximately 60 per cent of the phospholipids of the petroleum ether extract are choline-containing.   The molal ratios for the methanol eluate are very close to unity.   With the exception of a single value, the ratios ranged from 0.94 to 1.04; the average was 0.99.   Since the choline to phosphorus ratio in a pure choline-containing phospholipid is unity, the observed ratios for the methanol eluate provide evidence that a satisfactory separation of the C. C. from the N. C. C. phospholipids of the liver had been effected by the procedure used here.

The choline, P^{31}, and P^{32} contents of both the petroleum ether extracts and the methanol eluates were expressed *as amounts per gm. of liver.*

The amounts of C. C. phospholipid phosphorus per gm. of liver recorded in Table I were calculated as the product,

$$\text{Mg. P of petroleum ether extract} \times \text{molal ratio,} \quad \frac{\text{choline}}{\text{phosphorus}} \quad \text{of same extract}$$

TABLE I

Choline-Containing and Non-Choline-Containing Phospholipids of Liver of Dog

Experi-ment No.	Dog No.	Hrs. after P^{32} injection	Choline to P^{31} molal ratio		Choline-containing phospho-lipid P^{31}	Non-choline-containing phospho-lipid P^{31}
			Petroleum ether extract	Methanol eluate		
					mg. per gm. liver	*mg. per gm. liver*
1	Choline-treated, 5-86A	6	0.62	0.95	0.66	0.40
		12	0.56	0.96	0.61	0.48
		18	0.58	1.03	0.58	0.42
		24	0.58	1.04	0.61	0.44
	Control, 5-86B	6	0.55	0.97	0.61	0.51
		12	0.54	0.99	0.59	0.49
		18	0.50	0.99	0.60	0.60
		24	0.52	1.00	0.60	0.56
2	Choline-treated, 5-98D	6	0.65	0.95	0.80	0.42
		12	0.63	0.95	0.78	0.46
		18	0.62	0.94	0.86	0.53
		24	0.66	0.95	0.88	0.45
	Control, 5-98C	6	0.57	0.94	0.60	0.45
		12	0.57	0.95	0.57	0.43
		18	0.62	0.99	0.61	0.38
		24	0.60	0.98	0.80	0.53
3	Choline-treated, 28R	6	0.56	0.87	0.70	0.55
		12	0.58	0.95	0.76	0.55
		18	0.57*	0.99*	0.77*	0.59*
		18	0.57*	0.96*	0.79*	0.59*
	Control, 28S	6	0.57	0.95	0.64	0.48
		12	0.62	0.95	0.67	0.51
		18	0.52*	0.97*	0.67*	0.63*
		18	0.52*	0.94*	0.64*	0.59*

* Taken from different lobes of the same liver.

The amounts of N. C. C. phospholipid phosphorus recorded in Table I represent the difference between the total phospholipid phosphorus per gm. of liver and the C. C. phospholipid phosphorus per gm. of liver.

Since all phospholipids of the methanol eluate are choline-containing, the specific activity of the C. C. phospholipid phosphorus was derived

from the ratio of $P^{32}:P^{31}$ of the methanol eluate, the P^{32} being expressed as a percentage of the injected P^{32} and the P^{31} in mg.

Since the elution of C. C. phospholipids by methanol is not complete,[3] a correction was applied to the value obtained for the P^{32} content of the methanol eluate before making the calculation for specific activity of the N. C. C. phospholipid phosphorus. Thus the value for *total* C. C. phospholipid P^{32} is obtained by multiplying the P^{32} content of the methanol eluate by the ratio, choline content of the petroleum ether extract to choline content of the methanol eluate. Then the N. C. C. phospholipid P^{32} is the difference between the P^{32} content of the petroleum ether extract and the P^{32} content of the methanol eluate corrected as described above. The specific activity of the N. C. C. phospholipid phosphorus is equal to the

TABLE II

Uniformity of Phospholipid Content in Various Lobes of Liver

Dog No.		Left main lobe	Right middle lobe	Left middle lobe	Right main lobe
2-2	Phospholipid P^{31}, *mg. per gm. liver*	1.12	1.05	1.10	1.25
	Counts phospholipid P^{32} per gm. / Mg. phospholipid P^{31} per gm.	0.014	0.015	0.015	0.014
2-9	Phospholipid P^{31}, *mg. per gm. liver*	1.44	1.50	1.37	1.45
	Counts phospholipid P^{32} per gm. / Mg. phospholipid P^{31} per gm.	0.025	0.024	0.024	0.026

ratio, N. C. C. phospholipid P^{32} per mg. of N. C. C. phospholipid P^{31}, the P^{32} being expressed as a percentage of the injected P^{32}.

Since the amounts of phospholipid P^{31} and phospholipid P^{32} contained in small liver samples were used as an index of phospholipid activity of the whole liver, it became necessary to determine the uniformity of their contents among the various liver lobes. Two dogs were injected intraperitoneally with radioactive inorganic phosphate. 6 hours later they were anesthetized with pentobarbital, and small samples (3 to 4 gm.) of each of the four main lobes were excised and transferred to flasks containing 95 per cent ethyl alcohol. Petroleum ether extracts of these liver samples were then prepared in the usual manner (3) and these analyzed for phospholipid P^{31} and phospholipid P^{32}. The results recorded in Table II show that phospholipid is fairly evenly distributed throughout the liver. Moreover, the values obtained for the ratio of radiophospholipid phosphorus to total phospholipid phosphorus are practically the same throughout the various lobes. These findings leave no doubt that the phospholipid activity of a small sample of liver is a reliable index of the liver's total activity.

[3] The amount eluted was never less than 90 per cent.

Results

The results of three experiments are presented in Table I and Figs. 1 to 3. In each the phospholipid turnover in the liver of a choline-treated dog is compared with that in a dog that received no choline. Care was taken to keep the dietary treatment of the two dogs identical during the 2 to 3 weeks preceding the injection of the radiophosphorus, and during the actual course of the experiment their treatment differed only in a single respect, *i.e.* the enteral administration of choline.
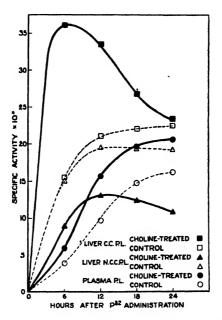

FIG. 1. The effect of choline on the specific activity-time relations of C. C. and N. C. C. phospholipid phosphorus of liver (Experiment 1).

The specific activities of the C. C. phospholipid phosphorus shown in Figs. 1 to 3 represent the ratio of the percentage of injected P^{32} recovered as C. C. phospholipid per gm. of liver to the mg. of P^{31} found as C. C. phospholipid per gm. of liver. The specific activities of the N. C. C. phospholipid phosphorus were obtained in a similar manner.

Experiment 1—In the control dog (No. 5-86B) the "specific activity-time" curves were found to be quite similar for the C. C. and the N. C. C. phospholipids of the liver (Fig. 1). Thus at 6 hours after the injection of P^{32} the specific activities of the C. C. and the N. C. C. phospholipid phosphorus were respectively 15.5×10^{-3} and 15.0×10^{-3}, and even at 24 hours the values for these two liver fractions were 22.5×10^{-3} and 19.2×10^{-3}.
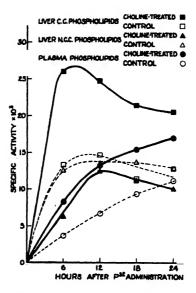
FIG. 2. The effect of choline on the specific activity-time relations of C. C. and N. C. C. phospholipid phosphorus of liver (Experiment 2).
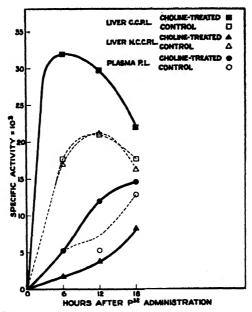
FIG. 3. The effect of choline on the specific activity-time relations of C. C. and N. C. C. phospholipid phosphorus of liver (Experiment 3).

The specific activity values of plasma phospholipid phosphorus of this dog were lower than those for either liver fraction during the four intervals studied.

The administration of choline produced a profound effect on the specific activities of the C. C. phospholipid phosphorus of the liver of Dog 5-86A. As early as 6 hours, the specific activity of this fraction rose to 36.1×10^{-3}; this value was 4 times that of the N. C. C. phospholipid phosphorus. Although at 24 hours the specific activity of the C. C. phospholipid phosphorus of the liver had dropped to 23.4×10^{-3}, the latter was twice as high as the value for the specific activity of the N. C. C. phospholipid phosphorus.

Experiment 2—The results of this experiment confirm those of the preceding experiment. Thus in the control dog (No. 5-98C) the two values found at each time interval for the specific activities of the C. C. and the N. C. C. phospholipid phosphorus differed very little (Fig. 2). But in the choline-treated dog (No. 5-98D) the curves depicting the specific activity-time relations of these two types of phospholipids were not the same; at the 6 hour interval the specific activity for the C. C. and the N. C. C. phospholipid phosphorus was respectively 26.0×10^{-3} and 6.2×10^{-3}, and at the other three intervals the values for the C. C. were twice those for the N. C. C. phospholipids. The values for the specific activities of plasma phospholipids were higher in the choline-treated than in the control dog.

Experiment 3—Here again the specific activities of the C. C. and the N. C. C. phospholipid phosphorus of the liver at each time interval were practically the same in the control dog (No. 28S) (Fig. 3). In the choline-treated dog (No. 28R) the values for the specific activities of the C. C. phospholipid phosphorus were extraordinarily high as compared with those of the N. C. C. phospholipid phosphorus.

<center>DISCUSSION</center>

Since ingested choline increased the rate of change in the specific activity of plasma phospholipid phosphorus (1), nearly all of which is the C. C. type (7), and moreover since the site of formation of plasma phospholipids is the liver (2), it seemed a reasonable inference that the action of choline upon the liver would be concerned with C. C. phospholipids. This view is fully borne out by the observations presented here. A comparison of the specific activity-time relations of the C. C. phospholipid phosphorus of the liver in the choline-treated and in the control dogs of each experiment suggests that the turnover of this particular fraction of liver phospholipid is increased when choline is ingested.

It is interesting that ingested choline did not increase the specific activity of the N. C. C. phospholipid phosphorus of the liver. On the contrary,

the administered choline depressed its specific activity. In all three experiments, the specific activities for N. C. C. phospholipid phosphorus were lower in the choline-treated than in the control dog.

Criteria that serve to determine whether an organ can be a source of plasma phospholipids have been considered elsewhere (2, 8). It was pointed out that during the early intervals after the single administration of a labeling agent the specific activity of the immediate precursor of a compound must be greater than that of the compound itself. In all six dogs the specific activities of the C. C. phospholipid phosphorus of the liver were higher than those of plasma phospholipid phosphorus throughout the entire period of observation. This finding reinforces earlier evidence showing that practically all phospholipids of plasma are formed in the liver (2).

SUMMARY

1. Dogs were injected with radioactive phosphorus, and the specific activities of the choline-containing and non-choline-containing phospholipids of their livers were measured.

2. The "specific activity-time" curves of the choline-containing and the non-choline-containing phospholipid phosphorus of the liver are quite similar in untreated dogs.

3. A single ingestion of 300 mg. of choline per kilo of body weight greatly increases the specific activities of choline-containing phospholipid phosphorus of the liver.

4. Ingested choline decreases the specific activities of the non-choline-containing phospholipid phosphorus of the liver.

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THE DISTRIBUTION PATTERN OF FATTY ACIDS IN GLYCERIDES OF MILK FAT

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To understand the chemical nature of natural fats, one must know the fatty acid composition, the distribution pattern of the fatty acids among the glycerides, and the molecular configuration of the individual glycerides. Rather complete knowledge of the fatty acid composition of most natural fats is now available, or can be obtained readily by methods now in use. For determining specific molecular configuration, methods involving x-ray diffraction and similar techniques are being developed.

A logical, but not proved, distribution pattern of the fatty acids among the glycerides can be obtained from the fatty acid composition, provided the percentage of saturated glycerides has also been determined. Occasionally hydrogenation, to establish the location of the unsaturated constituents, is useful.

Hilditch (1), using the above technique, has proposed a general distribution pattern for the fatty acids in natural fats. He believes that fatty acids follow a "rule of even distribution." This term, "even distribution," is indefinite and subject to interpretation. Hilditch states, "Any individual triglyceride molecule tends toward maximum heterogeneity in its composition. This [the distribution of fatty acids according to their relative proportions as evenly as possible among all the glyceride molecules] is, in fact, the main principle which seems to be operative in the structure of natural fats." The authors interpret these statements to mean that the component fatty acids are distributed as widely as possible among the various glycerides. Hilditch further points out that in certain animal fats, notably milk fats, the fatty acids are even more widely distributed among the glycerides than in most vegetable fats; this observation extends the principle of heterogeneity of glyceride structure.

According to Longenecker and his associates (2–4), the fatty acids are randomly distributed, and the occurrence of each type of glyceride can be calculated from suitable probability equations. Random distribution requires that all possible combinations of glycerides be present according to the relative proportions of the component fatty acids. Cow's milk fat with a relatively high content of oleic and palmitic acids should, then, contain significant quantities of triolein and tripalmitin.

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TABLE I

Distillation and Analyses of Milk Fat and Fractions

Fraction	Steam-volatile acids		"Solid" methyl esters			"Liquid" methyl esters		
	Weight	Saponification equivalent	Weight	Saponification equivalent	I No. (Hanus)	Weight	Saponification equivalent	I No. (Hanus)
	gm.		*gm.*			*gm.*		
−7° ppt.	1.17	89.0	0.88	200.7		This fraction consid-		
			0.78	216.7		ered as containing		
			6.18	238.7	0.89	only "solid" acids		
			2.03	242.2				
			1.23	272.0				
			1.93	272.2				
			8.03	273.0	1.28			
			9.38	272.7	1.03			
			2.88	275.5	0.69			
			3.68	294.0	28.03			
			7.03	298.0	18.50			
			3.80	304.0	15.33			
			2.40	331.5	17.27			
−13° ppt.	0.61	116.0	0.62	207.2		2.41	182.0	11.43
	1.82	126.4	2.01	243.3		0.68	197.2	7.00
	0.98	140.0	1.78	254.7		2.03	206.6	6.88
	1.68	140.6	5.98	271.0	0.76	0.81	219.5	8.00
	2.76	152.2	20.18	270.5	0.94	6.78	240.3	9.53
	0.49	161.2	3.38	270.4	4.06	2.48	247.5	8.73
	Ether and water		1.36	267.6	21.63	2.13	253.0	17.58
	titrations cal-		7.98	294.8	21.02	3.16	266.5	22.16
	culated as		4.10	298.0	9.37	1.58	272.9	28.10
	butyric acid,		3.50	321.3	15.65	1.28	277.0	58.80
	2.29 gm.					7.78	291.2	88.72
						4.38	288.8	92.55
						2.90	303.2	92.00
						3.20	316.5	91.50
−23° ppt.	1.05	87.6	0.74	215.5		0.57	179.0	
	2.06	106.7	3.00	234.8	4.48	1.70	181.9	12.75
						2.13	209.6	6.67
	0.75	122.3	1.71	249.0	3.06	0.65	229.0	7.00
	0.25	167.4	4.71	267.2	1.68	9.68	241.3	8.04
	Ether and water		4.08	269.0	1.52	1.08	255.5	11.04
	titrations cal-		9.78	269.5	0.63	2.05	258.1	21.86
	culated as		7.98	271.0	0.63	3.30	271.5	22.15
	butyric acid,		3.78	270.9	2.64	2.40	284.3	68.50
	1.77 gm.		2.38	284.0	25.63	5.96	286.0	91.84
			6.98	292.8	22.53	2.58	294.2	89.50
			2.00	287.0	9.44	3.10	290.1	89.45
			1.60	329.5,	27.25	3.70	317.4	100.22
−53° ppt.	2.43	106.9	0.68	192.4		2.28	181.4	8.03
	1.87	113.8	0.73	214.4		0.98	196.3	9.58
	0.94	125.0	2.63	240.2	4.30	2.03	206.7	7.18
						0.98	218.6	7.86

TABLE I—*Concluded*

Fraction	Steam-volatile acids		"Solid" methyl esters			"Liquid" methyl esters		
	Weight	Saponification equivalent	Weight	Saponification equivalent	I No. (Hanus)	Weight	Saponification equivalent	I No. (Hanus)
	gm.		*gm.*			*gm.*		
−53° ppt.— *Continued*	Ether and water titrations calculated as butyric acid, 2.48 gm.		2.28	250.0	5.00	7.00	241.1	8.67
			1.53	252.2	4.44	2.08	242.3	6.39
			6.73	266.0	2.96	1.06	246.5	10.41
			4.73	267.9	2.68	1.48	256.2	23.57
			15.03	270.3	0.86	2.91	265.6	30.64
			2.08	281.2	22.61	1.48	268.8	40.70
			2.93	293.9	41.78	4.03	291.7	80.47
			5.51	298.6	31.85	1.68	296.0	82.50
			3.00	298.7	20.15	6.37	295.7	93.90
			4.60	311.5	24.44	1.80	298.1	93.60
						2.60	298.2	91.90
						3.90	309.2	100.60
−53° filtrate	1.12	102.5	This fraction considered as containing only "liquid" acids			0.79	186.1	
	1.40	104.8				0.78	195.0	
	1.73	108.0				0.98	209.1	9.48
	0.93	108.5				0.72	216.2	12.00
	2.05	114.0				2.63	240.7	20.88
	0.60	116.6				1.65	244.5	18.56
	0.56	132.6				1.35	260.9	22.86
	1.68	141.3				4.68	268.0	22.28
	0.60	167.2				2.98	270.7	21.43
	Ether and water titrations calculated as butyric acid, 4.24 gm.					1.64	276.5	42.28
						1.98	291.8	72.65
						5.91	296.4	90.20
						5.72	297.8	92.60
						7.71	295.5	87.85
						3.50	298.6	95.70
						2.26	299.0	95.60
Milk fat	1.25	91.3	0.61	207.3		1.03	180.8	11.23
	0.93	111.0	2.58	234.1	3.34	0.98	197.5	13.58
	1.40	121.4	1.88	241.2	1.33	0.80	195.8	10.00
	0.39	127.5	2.83	259.3	2.11	1.66	214.4	6.88
	0.46	139.4	9.48	267.2	1.16	0.78	214.5	8.00
	0.98	141.5	14.55	270.4	1.17	6.28	240.2	11.07
	Ether and water titrations calculated as butyric acid, 2.73 gm.		2.38	279.0	14.11	2.43	248.5	14.05
			1.95	291.7	32.13	2.06	251.4	24.01
			6.38	291.7	30.46	2.61	262.8	33.10
			4.00	295.0	18.33	1.62	259.1	37.01
			3.90	309.1	22.70	1.53	272.5	70.05
						3.63	293.3	91.95
						4.73	293.3	95.05
						4.71	290.7	94.95
						3.73	290.9	94.45
						1.60	289.4	95.00
						3.20	315.0	97.90

This paper will present data showing the probable distribution pattern in milk fat (results obtained by subjecting milk fat to fractionation from the solvent), followed by analyses of the separate fractions to determine the component fatty acids and the fully saturated glycerides.

EXPERIMENTAL

Milk fat was fractionated from the solvent as previously described (5) by freezing out portions at $-7°$, $-13°$, $-23°$, $-53°$, and the filtrate remaining at $-53°$. Milk fat was dissolved in Skellysolve A and cooled

TABLE II

Molar Percentage of Fatty Acids in Milk Fat Fractions

		−7° ppt.	−13° ppt.	−23° ppt.	−53° ppt.	−53° filtrate	Milk fat
Saturated	C_4	2.5	4.6	8.7	7.9	9.3	9.2
	C_6		2.9	4.0	5.8	6.9	2.8
	C_8		6.6	1.3	1.7	2.4	2.7
	C_{10}	1.2	4.9	4.0	5.5	4.5	3.5
	C_{12}	4.5	3.5	4.2	4.7	3.7	5.2
	Total	8.2	22.5	22.2	25.6	26.8	23.4
	C_{14}	16.6	11.3	14.6	16.5	7.4	14.8
	C_{16}	42.1	36.2	38.1	24.0	14.0	27.2
	C_{18}	21.1	9.3	5.2	8.4	6.8	8.5
	C_{20}	3.8	3.1	1.0	1.4		1.2
	C_{22}	0.8					
	Total	25.7	12.4	6.2	9.8	6.8	9.7
Total		92.6	82.4	81.1	75.9	55.0	75.1
Unsaturated	C_{10}		0.2	0.1	0.3	1.4	0.3
	C_{12}		0.2	0.1	0.2	0.4	0.2
	C_{14}	0.1	0.9	1.2	1.4	1.8	1.5
	C_{16}	0.4	3.9	4.5	2.7	4.6	5.2
	C_{18}	6.1	10.6	10.7	17.2	30.0	15.3
	C_{20}	0.8	1.3	1.9	1.8	0.8	0.7
	Linoleic		0.5	0.4	0.5	6.0	1.7
Total		7.4	17.6	18.9	24.1	45.0	24.9

to $-7°$, the precipitate was filtered off, and the filtrate lowered to $-13°$, at which point another precipitate was removed. This procedure was repeated through $-23°$ and $-53°$. The fatty acid composition of the glyceride fractions was determined by ester fractionation, as described

previously (6). Table I shows the analytical data for the distillates. Table II shows the fatty acid composition as calculated from these data.

The fractions were subjected to mild oxidation, potassium permanganate in acetone being used, as described by Hilditch and Lea (7) and Hilditch (1). The oxidized unsaturated portions were removed by suitable washing, and the fully saturated glycerides determined. The proportions of potassium permanganate needed for such oxidations, as well as the reaction time,

TABLE III

Percentage of Fully Saturated Glycerides Present in Milk Fat Fractions

Fraction	Experimental per cent fully saturated glycerides		Limiting minimum value from composition*	Per cent calculated on basis of random distribution†
	Weight	Moles	Moles	Moles
−7° ppt................	75.3	75.4	77.8	79.4
−13° "	43.4	40.5	47.2	55.8
−23° "	44.2	40.3	43.3	53.3
−53° "	26.2	25.8	27.7	43.6
−53° filtrate............	3.8	4.6	0	16.6
Milk fat................	33.4	31.3	25.3	41.8

* This value is calculated by assuming that the unsaturation is distributed one fatty acid to a glyceride. Thus in the −7° precipitate with 7.4 moles of unsaturated acids (Table II) there could be a maximum of $3 \times 7.4 = 22.2$ moles of unsaturated glycerides and, therefore, a minimum of 77.8 moles of fully saturated glycerides.

† The calculation of occurrence in random distribution is governed by the following equation (H. E. Longenecker, University of Pittsburgh, personal communication). Molar percentage of any specific glyceride $= n \times ((a \times b \times c)/100^3) \times 100$, where $n =$ frequency of the glyceride occurrence in random distribution and a, b, and $c =$ molar percentage of the fatty acids in positions 1, 2, and 3 of the glycerol respectively. For example, −7° precipitate has 92.6 per cent of saturated acids, and $n = 1$; then the molar percentage of fully saturated glycerides in the −7° precipitate $= 1 \times (92.6 \times 92.6 \times 92.6/100^3) \times 100 = 79.4$.

will vary with the nature of the fat under oxidation. Considerable experimentation was required to obtain satisfactory results, namely complete removal of the unsaturated portions as indicated by an iodine value of less than 1, without letting excessive oxidation destroy the saturated portions. Table III shows the percentage of fully saturated glycerides, together with limiting minimum values calculated from the fatty acid composition given in Table II. On the whole, the experimental values for the fully saturated glycerides are somewhat low, several being less than the calculated limiting value. Probably, however, this situation should be expected, since the laboratory manipulations necessary to remove the monoazelaoglycerides (that is, the acidic products from monooleoglycerides)

undoubtedly cause slight losses of the fully saturated glycerides also. Since the work was done with exceptional care, the authors are certain that the experimental error was not sufficient to lower the true results by the difference between the experimental values and those calculated on the basis of random distribution (Table III, last column). This point will be discussed in greater detail later.

The fully saturated glycerides were first saponified and then esterified with methyl alcohol; and the methyl esters were fractionally distilled through an electrically heated, packed column, as described elsewhere (6). Table IV shows the mole percentage of fatty acids present in each.

TABLE IV

Fatty Acid Composition of Fully Saturated Glycerides in Milk Fat Fractions
The values are given in moles per cent.

No. of carbon atoms	−7° ppt.	−13° ppt.	−23° ppt.	−53° ppt.	−53° filtrate*	Milk fat
C_{4-12}	8.9	24.2	22.5	32.8	76.0	26.5
C_{14}	17.9	8.3	15.9	21.8	21.3	13.4
C_{16}	45.5	50.0	55.8	36.3	2.7	47.9
C_{18-22}	27.7	17.5	5.8	9.1		12.2

* The molar percentages of fatty acids in the fully saturated glycerides of the −53° filtrate are not considered reliable, because the small percentage of fully saturated glycerides (4.6 moles per cent) present necessitated the working up of a large mass of material in order to get a sufficient quantity for analysis. Considerable difficulty was experienced, furthermore, in washing acidic compounds from this sample after oxidation with permanganate. Very persistent emulsions were also encountered in washing this material.

DISCUSSION

The importance of these findings lies in the interpretation placed on them and in the considerations that follow as a consequence. In the authors' opinion, these results show that the distribution pattern of fatty acids in glycerides from milk fat tends more nearly to the pattern of *widest possible distribution* than to the pattern of *random distribution*. The values in Table III support this view. The experimental data for the fractions conform closely to the limiting minimum values, which represent the *widest possible distribution* of the unsaturated acids. This situation is probably to be expected, since any possible randomness in the entire fat would be obliterated by the selectivity practiced in preparing the fractions. The relative solubilities of different glycerides govern the fraction in which they appear. As the fractions show, the unsaturation is distributed as widely as possible.

It does not necessarily follow that the entire fat from which the fractions were separated should have the same distribution pattern, since the fractions represent selected glycerides. Judging from the "association ratios," the milk fat does follow the same *widest distribution* pattern as the fraction. The "association ratio" proposed by Hilditch (1) is the ratio between the saturated fatty acids and the unsaturated fatty acids in the mixed glycerides. The precipitated fractions ($-7°$, $-13°$, $-23°$, $-53°$) all have association ratios of almost exactly 2.0; and the filtrate fraction ($-53°$) has a ratio of 1.2. This means that essentially all the mixed glycerides in the precipitated fractions are of the disaturated type, while those of the filtrate are about equally divided between di- and monosaturated. Monosaturated glycerides would have a ratio of 0.5 and equal mixtures, therefore, would have a ratio of $(0.5 + 2.0)/2 = 1.25$. An average ratio of all the fractions, weighted on the basis of the molar proportions of each fraction in the entire fat, is 1.80. The association ratio of the milk fat is determined to be 1.76, calculated as follows:

There are 31.3 moles per cent of fully saturated glycerides in the milk fat; $100 - 31.3 = 68.7$ moles of mixed glycerides in the milk fat (Table III). There are 24.9 moles per cent of unsaturated fatty acids in the milk fat (Table II). $68.7 - 24.9 = 43.8$ moles of saturated fatty acids in the mixed glycerides. $43.8/24.9 = 1.76$ association ratio.

The agreement between these two ratios, 1.80 and 1.76, strongly supports the contention that milk fat has the same distribution pattern of saturated and unsaturated fatty acids. If there had been present more than the minimum number of monosaturated glycerides that could be accounted for on the basis of widest possible distribution from the percentage of unsaturated fatty acids, then the percentage of fully saturated glycerides would have to be higher than was found.

Granting that the unsaturated fatty acids in milk fat are distributed as widely as possible, one may logically assume the same distribution pattern for the saturated fatty acids. On the basis of this assumption, the following general scheme of glyceride types is proposed.

Simplified General Scheme of Glyceride Types—If one divides the number of moles of fatty acids (grouped for convenience C_{4-12}, C_{14}, C_{16}, C_{18-22}, and unsaturated) present in each fraction (Table II) by the highest common factor, the result is the minimum proportions of each. If the total of these is divided by 3 (fatty acids per glyceride), the result will be the number of simplest types that can be constructed from the component fatty acids. Below are given these calculations, together with the simplest glyceride types based on *widest possible distribution* of the fatty acids. No significance is attached to the positions here of the fatty acids within the glyceride

molecules; no data are presented that would justify any speculation on molecular configuration.

−7° Precipitate

Group	Moles per cent	Ratio
C_{4-12}	8.2	1
C_{14}	16.6	2
C_{16}	42.1	5
C_{18-22}	25.7	3
Unsaturated	7.4	1
		12 (Total)

$$CH_2O-$$
$$|$$
12 ÷ 3 = 4 general types in which ⊢ stands for the glyceryl radical CHO—
$$|$$
$$CH_2O-$$

C_{16}	C_{16}	C_{16}	C_{16}
C_{18-22}	C_{18-22}	C_{18-22}	C_{16}
C_{4-12}	C_{14}	Unsaturated	C_{14}

77.8 per cent fully saturated glycerides; 22.2 per cent unsaturated glycerides

−15° Precipitate

Group	Moles per cent	Ratio
C_{4-12}	22.5	4
C_{14}	11.3	2
C_{16}	36.2	7
C_{18-22}	12.4	2
Unsaturated	17.6	3
		18 (Total)

18 ÷ 3 = 6 general types

C_{16}	C_{16}	C_{16}	C_{16}	C_{16}	C_{16}
C_{4-12}	C_{4-12}	C_{4-12}	C_{14}	C_{18-22}	C_{16}
C_{14}	C_{18-22}	Unsaturated	Unsaturated	Unsaturated	C_{4-12}

47.2 per cent fully saturated glycerides; 52.8 per cent unsaturated glycerides

−25° Precipitate

Group	Moles per cent	Ratio
C_{4-12}	22.2	3
C_{14}	14.6	2
C_{16}	38.1	6
C_{18-22}	6.2	1
Unsaturated	18.9	3
		15 (Total)

15 ÷ 3 = 5 general types

$$\left[\begin{array}{l} C_{16} \\ C_{18-22} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{14} \\ C_{4-12} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{4-12} \\ C_{14} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{16} \\ \text{Unsaturated} \end{array}\right.$$

43.3 per cent fully saturated glycerides; 56.7 per cent unsaturated glycerides

$-53°$ Precipitate

Group	Moles per cent	Ratio
C_{4-12}	25.6	3
C_{14}	16.5	2
C_{16}	24.0	3
C_{18-22}	9.8	1
Unsaturated	24.1	3
		12 (Total)

$12 \div 3 = 4$ general types

$$\left[\begin{array}{l} C_{16} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{16} \\ C_{14} \\ C_{4-12} \end{array}\right. \quad \left[\begin{array}{l} C_{18-22} \\ C_{14} \\ \text{Unsaturated} \end{array}\right.$$

27.7 per cent fully saturated glycerides; 72.3 per cent unsaturated glycerides

$-53°$ Filtrate

Group	Moles per cent	Ratio
C_{4-12}	26.8	4
C_{14}	7.4	1
C_{16}	14.0	2
C_{18-22}	6.8	1
Unsaturated	45.0	7
		15 (Total)

$15 \div 3 = 5$ general types

$$\left[\begin{array}{l} C_{16} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{14} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{14} \\ C_{4-12} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{18} \\ \text{Unsaturated} \\ \text{Unsaturated} \end{array}\right. \quad \left[\begin{array}{l} C_{4-12} \\ \text{Unsaturated} \\ \text{Unsaturated} \end{array}\right.$$

0 per cent fully saturated; 100 per cent unsaturated

Glycerides of the types postulated for the different fractions would tend to form a fat mixture having the physical characteristics of the fractions actually obtained in practice. To illustrate this it would be possible from the fatty acid composition to have a glyceride of the type $\left[\begin{array}{l} C_{4-12} \\ C_{14} \\ \text{Unsaturated} \end{array}\right.$ in the $-7°$ precipitate. It is very doubtful that such a type would be present in this fraction; more logically, one might expect it to pass to one of the lower freezing fractions.

One can construct a hypothetical milk fat from these glyceride types, using increments of each fraction in proportion to the molar percentage

that each fraction represents; namely, 10.5 per cent for the $-7°$ precipitate, 20.5 per cent for the $-13°$ precipitate, 20.9 per cent for the $-23°$ precipitate, 23.8 per cent for the $-53°$ precipitate, and 24.3 per cent for the $-53°$ filtrate. The calculated percentage of fully saturated glycerides in this hypothetical milk fat, based on *widest possible distribution* of the fatty acids would be $(77.7 \times 0.105) + (47.2 \times 0.205) + (43.3 \times 0.209) + (27.7 \times 0.238) + (0 \times 0.243) = 33.1$ per cent, which compares well with 31.3 per cent, the experimental value determined from milk fat. This is further evidence that the fatty acids of milk fat tend toward *widest possible distribution*. Similarly, calculations can be made for the probable occurrence of other types of glycerides in a hypothetically constructed milk fat.

SUMMARY

1. Data are presented showing the fatty acid composition, the percentage of fully saturated glycerides, and the fatty acid composition of the fully saturated glycerides of milk fat fractions separated from the solvent.

2. The mole percentage of fully saturated glycerides found is as follows: $-7°$ precipitate 75.4 per cent, $-13°$ precipitate 40.5 per cent, $-23°$ precipitate 40.3 per cent, $-53°$ precipitate 25.8 per cent, $-53°$ filtrate 4.6 per cent, and original milk fat 31.3 per cent.

3. It is proposed, from a consideration of these values, that the fatty acids in milk fat tend to be distributed among the glycerides as widely as possible.

4. Patterns for the distribution of the fatty acids among the glycerides in the different fractions are postulated, based on the proposition that the fatty acids are distributed as widely as possible.

5. The hypothetical patterns are compared with the experimental data.

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STORAGE OF HENDECANOIC ACID IN THE WHITE RAT

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The fate of ingested fatty acids depends upon their chain length (number of carbon atoms per fatty acid). Lower fatty acids such as butyric and caproic acids are not stored as such (1) but are in part oxidized to ketone bodies (2). Available evidence indicates that they are not directly involved in the synthesis of higher fatty acids (3). Lower "odd carbon" fatty acids (possessing an odd number of carbon atoms) are in part oxidized to yield ketone bodies and carbohydrate precursors (4). On the other hand, the higher "even carbon" acids, exemplified by palmitic acid, are largely laid down in the fat depots, there to await interconversion or mobilization (5).

In order to study the fate of an "odd carbon" fatty acid of intermediate chain length, hendecanoic (undecylic) acid in the form of a glyceride was fed to rats and the fatty acids of the depots were partially separated as their lead salts, converted to the methyl esters, and fractionally distilled. The results point to the deposition of hendecanoic acid as such. As the methods employed were incapable of demonstrating the presence of small quantities of "odd carbon" fatty acids among the "even carbon" acids normally present, no decisive evidence as to the subsequent conversion of the hendecanoic acid could be secured.

EXPERIMENTAL

Young rats averaging 137 gm. in weight were taken from a stock diet, divided into two groups of eighteen rats, and placed for 6 weeks on a diet of commercial casein 25 parts, brewers' yeast 10 parts, Osborne and Mendel's salt mixture (6) 5 parts, cane sugar 35 parts, and glyceride 25 parts. The glyceride fed control Group A was coconut oil, while the glyceride fed experimental Group B was synthesized from purified hendecanoic acid (Eastman Kodak). The purification was carried out in a 45 inch column packed with a spiral wire gauze. It was found necessary to discard some 3 per cent of higher acids. The purified hendecanoic acid had an iodine number of 3 and an equivalent weight of 186. The glyceride was formed by heating glycerol and undecylic acid to 200° under nitrogen in the ratio of 1 mole of glycerol to 2 moles of undecylic acid. A partial vacuum of 200 mm. of Hg was employed. The temperature was held for 5 hours. A small amount of unesterified acid was removed by washing the petroleum ether solution of the glycerides with dilute potassium carbonate solution.

It may be worth recording that rats on a diet containing 25 per cent of the free fatty acids of coconut oil or of undecylic acid failed to grow, a result due, perhaps, to the lack of palatability of the diet.

The rats of Group A grew at an average rate of 1.2 gm. per day, while those of Group B gained an average of 0.9 gm. per day over the same period. After being anesthetized with sodium pentabarbital, the rats of Group B were bled and the stomach, intestines, spleen, and kidneys were removed. Each carcass was then dissolved in 250 cc. of cold 30 per cent KOH with occasional agitation over several days. The caustic solution was kept covered with a layer of petroleum ether. The calcareous material was separated from the supernatant solution and discarded. The lipids were obtained for analysis by acidification of the pooled basic solutions and subsequent extraction with petroleum ether. The mixture of glycerides, free fatty acids, and non-saponifiable matter obtained in this manner was saponified. The fatty acids weighed 270 gm. They were separated into solid and liquid fractions by the Pb soap-alcohol method in which 230 gm. of lead acetate trihydrate and 20 volumes of EtOH were used for recrystallization at 15° and an equal volume for washing. The recrystallization and washing were repeated, 0.5 per cent acetic acid in alcohol being used for the recrystallization. The Pb soaps of the solid acids weighed 117 gm. They were esterified in 10 volumes of MeOH containing 7 per cent sulfuric acid. The MeOH solution was diluted with 2 volumes of water and extracted with ether to remove the esters. The ethereal solution was extracted with dilute potassium carbonate, which removed 1.5 gm. of free acid. This was reesterified and added to the main fraction. The weight of the methyl esters of the solid acids was 76 gm. The Pb soaps of the liquid acids were freed of EtOH by repeated additions and removal *in vacuo* of methyl alcohol. The methyl esters were prepared as before. The separated esters were extracted with dilute potassium carbonate solution and 8 gm. of unesterified acid were removed. This was reesterified and added to the main fraction. The yield of liquid esters was 199 gm.

The esters were fractionated in a modified Fenske column with an internal diameter of 18 mm. packed with single turn glass helices for 45 inches. The column has an efficiency of approximately forty theoretical plates. Iodine numbers were determined on each fraction by the method of Wijs. In determining the equivalent weight of the acids in a given fraction, the free acid was isolated and a weighed amount, from 100 to 300 mg.,was titrated with standard 0.1 N KOH in 10 cc. of neutral isopropyl alcohol solution containing a trace of phenolphthalein.

Calculations and Results

The weights of the various ester fractions with corresponding iodine number of the methyl ester and neutral equivalent of the fatty acid are

shown in Table I. Values are also shown for the estimated chain length composition of each fraction, calculated (7) on the assumption that hendecanoic acid is the only "odd carbon" fatty acid present. No attempt is made to assign to individual components the unsaturation indicated by the iodine numbers. The data in Table I yield the following figures for the percentage composition of the fatty acid mixture from the depots: 0.3 C_{10},

TABLE I

Analysis of "Solid" and "Liquid" Methyl Ester Mixtures from Fatty Acids of Rats Fed Undecylic Acid

Fraction No.	Weight	Equivalent weight of acid	I No. of ester	Estimated composition (weight)	
	gm.			*gm.*	
S-1	4.00	187.6	0.8	3.6 C_{11},	0.4 C_{12}
S-2	1.22	198.3	0.6	0.2 "	1.0 "
S-3	1.41	216.9	0.6	0.6 C_{12},	0.8 C_{14}
S-4	2.06	235.2	0.5	1.6 C_{14},	0.5 C_{16}
S-5	3.87	245.0	0.5	1.6 "	2.3 "
S-6	28.79	255.0	0.6	1.2 "	27.6 "
S-7	11.00	257.7	9.6	10.5 C_{16},	0.5 C_{18}
S-8	4.88	267.9	12.1	2.9 "	2.0 "
S-9	4.30	280.6	23.6	0.4 "	3.9 "
S-10	3.84	283.4	21.8	3.9 C_{18}	
S-11	2.83	286.0	24.0	2.7 "	0.2 C_{20}
L-1	0.25		8.4		
L-2	2.17	181.7	3.5	0.7 C_{10},	1.5 C_{11}
L-3	44.50	186.8	2.8	42.9 C_{11},	1.6 C_{12}
L-4	8.83	186.3	9.1	8.8 "	
L-5	3.75	201.4	14.9	3.7 C_{12},	0.1 C_{14}
L-6	4.60	234.9	44.9	3.5 C_{14},	1.1 C_{16}
L-7	4.01	252.8	71.2	0.2 "	3.8 "
L-8	11.71	256.1	70.0	11.7 C_{16}	
L-9	5.56	269.2	80.5	2.6 "	3.0 C_{18}
L-10	4.35	279.0	98.4	0.5 "	3.9 "
L-11	13.89	281.2	103.8	0.5 "	13.4 "
L-12	19.75	281.3	104.0	0.6 "	19.1 "
L-13	34.42	281.8	101.3	34.4 C_{18}	
L-14	9.31	283.0	98.6	9.1 "	0.2 C_{20}
L-15	6.24	286.3	111.6	5.3 "	0.9 "

23.9 C_{11}, 2.8 C_{12}, 3.5 C_{14}, 25.4 C_{16}, 43.6 C_{18}, 0.5 C_{20}. From the corresponding figures for "synthetic" rat fat, similarly calculated from analogous data (7) from animals on a fat-low diet, namely 0.1 C_{12}, 3.1 C_{14}, 42.3 C_{16}, 53.0 C_{18}, 1.3 C_{20}, it seems clear that extensive deposition of hendecanoic acid must have occurred. No evidence was found to indicate conversion of major amounts of hendecanoic acid to higher "odd carbon" fatty acids. However, more precise methods of fractionation or analysis are evidently necessary for

a decisive answer to the question of synthesis of higher "odd carbon" fatty acids from ingested hendecanoic acid.

The melting point of the free fatty acid from Fraction L-3 was observed to be identical with that of pure hendecanoic acid.

SUMMARY

Hendecanoic (undecylic) acid may be stored by the rat to the extent of 24 per cent of the depot fatty acids.

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A MODIFIED METHOD FOR THE QUANTITATIVE DETERMINATION OF THE THYMOL TURBIDITY REACTION OF SERUM

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It was observed by Maclagan that turbidity is produced in a barbital buffer saturated with thymol when specimens of serum from patients with diseases of the liver are added (1, 2). The degree of turbidity apparently varied with the degree of hepatic parenchymal damage. An analysis of the precipitate demonstrated that it contained globulin, phospholipid, cholesterol, and thymol. The mechanism of the reaction is unexplained, but there is some evidence to indicate that the reaction may be due to an abnormal globulin released into the blood stream in the presence of liver damage. The determination of thymol turbidity had been found useful in estimating the degree of hepatic damage in patients with diseases of the liver and in pathological states experimentally produced in animals.

In the original technique the amount of turbidity was estimated by visual comparison with the gelatin standards of Kingsbury *et al.* (3), which were devised for the estimation of urinary albumin. This method is subject to all of the usual criticisms of visual methods for colorimetric and nephelometric measurements. In the presence of hemolysis and of increased concentrations of bilirubin in the serum, accurate measurements of turbidity may be difficult to obtain. Moreover, the preparation and maintenance of adequate gelatin standards are associated with some difficulty. In order to obtain objective and more accurate quantitative measurement of turbidity, and to permit rigid standardization of the technique, the method has been modified in this laboratory so that turbidimetric determinations may be made in the spectrophotometer with a suspension of barium sulfate as a standard.

Method

Reagents—

Thymol-barbital buffer. A barbital buffer of pH 7.8, saturated with thymol, is prepared as described by Maclagan (2). 1.03 gm. of sodium

barbital, 1.38 gm. of barbital, and 3 gm. of powdered thymol crystals are placed in a 1000 cc. Erlenmeyer flask. 500 cc. of distilled water are added and the solution heated to the boiling point. The flask is removed from the flame and the contents mixed well by shaking. The solution is cooled to room temperature. On cooling, the solution becomes turbid. A small quantity of powdered thymol crystals is added and the solution again mixed by shaking. The flask is stoppered and is permitted to remain at room temperature overnight. Thymol crystals form at the bottom of the flask. After standing overnight, the solution is mixed once again by shaking, and is freed of crystalline deposit by filtration. The clear solution is used as the reagent and may be kept at room temperature indefinitely.

Barium chloride solution, 0.0962 N.

Sulfuric acid, 0.2 N.

Procedure

The test is carried out by adding 0.05 cc. of serum to 3.0 cc. of thymol-barbital buffer in a 10 × 75 mm. cuvette. The contents of the cuvette are mixed well and after 30 minutes turbidity is determined in the Coleman Junior spectrophotometer at a wave-length of 650 mμ. The galvanometer is adjusted to 100 per cent transmission with a blank containing 3.0 cc. of thymol-barbital buffer.

The turbidity of a given reaction is expressed in units derived from a standard curve prepared by use of barium sulfate suspensions. The turbidity standard is prepared by diluting 3.0 cc. of 0.0962 N barium chloride solution to volume in a 100 cc. volumetric flask by the addition of 0.2 N sulfuric acid at 10°. At this temperature the particle size of the precipitated barium sulfate is such that a comparatively stable suspension results. A 10 unit turbidity standard is prepared by adding 1.65 cc. of 0.2 N sulfuric acid to 1.35 cc. of the barium sulfate suspension in a 10 × 75 mm. cuvette. Similarly, a 20 unit standard is prepared by adding 0.3 cc. of 0.2 N sulfuric acid to 2.7 cc. of the barium sulfate suspension. These standards were chosen because they gave turbidimetric readings nearly equivalent to those obtained by Maclagan using visual comparison with gelatin standards. At room temperature there is some tendency for the barium sulfate suspension to settle out. For this reason cuvettes should be well shaken just before readings are made in the spectrophotometer. If a cuvette containing 3.0 cc. of distilled water is used as a blank, there is a straight line relationship between the optical density of various dilutions of the barium sulfate standard at 650 mμ (Fig. 1).

EXPERIMENTAL

It was necessary to determine the wave-length at which turbidity measurements could be made with least interference from those substances

in blood which absorb in the range of 400 to 700 mμ. Two substances which might be expected to interfere are hemoglobin resulting from hemolysis of red blood corpuscles and bilirubin which may occur in high concentration in the serum of patients with hepatic disease. In Fig. 2 the absorption
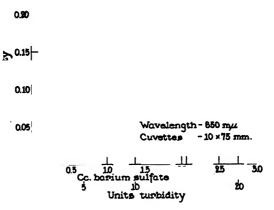
FIG. 1. Turbidity of barium sulfate suspensions as determined in the spectro-photometer.
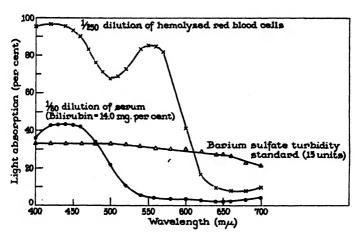
FIG. 2. Absorption spectra of a solution of hemolyzed red blood cells, a dilution of serum of high bilirubin content, and a barium sulfate turbidity standard.

spectrum of a turbidity standard of 15 units is compared with the absorption spectra of a 1:250 dilution of hemolyzed red blood cells and of a 1:60 dilution of serum containing 14.0 mg. per cent of bilirubin. It will be seen that turbidity determinations done at 650 mμ are relatively free of interference from light absorption due to bilirubin and hemoglobin. This wave-length is far removed from the absorption maxima of these com-

pounds. The concentration of hemoglobin in the solution tested far exceeds the amount that might be present in a 1:60 dilution of serum, even in the presence of gross hemolysis. At 650 mμ, light absorption by serum containing as much as 14.0 mg. per cent of bilirubin is insignificant when diluted as in the test. Therefore, it seemed that this wave-length was the one best adapted for turbidimetric determinations under the conditions of the thymol turbidity reaction.

The effectiveness of a procedure of this type is dependent upon rigorous standardization. The gelatin standards of Kingsbury *et al.* (3) contain a suspension of formalazin and are standardized against solutions of albumin precipitated with 3 volumes of 3 per cent sulfosalicylic acid. These standards are not easily reproducible and may change somewhat on standing. Attempts were made to obtain turbidity standards by the precipitation of solutions of albumin with 3 volumes of 3 per cent sulfosalicylic acid. Crystallized egg albumin prepared by precipitation with sodium sulfate (4), crystalline bovine albumin,[1] and human albumin[2] in concentrations varying from 0.05 to 0.2 gm. per cent were used. The concentration of albumin was determined by estimation of the protein nitrogen by the micro-Kjeldahl technique. There was a straight line relationship between the optical density at 650 mμ and varying concentrations of albumin. However, it was found that preparations of albumin from different sources, prepared by different methods, and probably characterized by varying degrees of denaturation failed to duplicate standard curves obtained with other albumin preparations. This is demonstrated in Fig. 3, in which the turbidimetric determinations of three different albumin preparations precipitated with sulfosalicylic acid are compared. For these reasons it became apparent that an albumin standard was wholly inadequate and another type of standard was sought. The barium sulfate standard of Wadsworth (5) which is used for measuring turbidity of bacterial suspensions was found to be a sufficiently stable and reproducible standard. It was adapted for use in this procedure, as described above.

The thymol turbidity reactions of sera from forty-six normal subjects were compared with those of sera obtained from 83 patients early in the course of acute infectious hepatitis. The results are compared in Table I. The highest turbidity value in the group of normal subjects was 4.7 units and the mean for this group was 2.66 units. There was a marked increase in thymol turbidity in the presence of liver damage produced by infectious hepatitis. The mean thymol turbidity reaction in the group of patients with infectious hepatitis was 17.03 units. Only one of the 83

[1] Armour and Company.
[2] Plasma Fractionation Laboratory at Harvard University.

patients had reactions of less than 5.0 units, which in the experience of this laboratory represents the high limit of normal. During convalescence from the disease there was a gradual decrease from initial high thymol turbidity to results that were in the normal range. Similar increased turbidity values
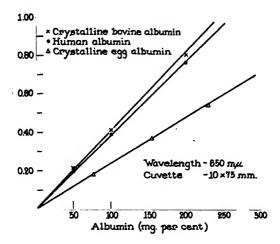
FIG. 3. Comparison of turbidity of varying concentrations of bovine albumin, human albumin, and egg albumin precipitated with sulfosalicylic acid.

TABLE I

Results of Thymol Turbidity Reaction in Forty-Six Normal Subjects and in 83 Patients with Infectious Hepatitis

Subject		Units of thymol turbidity								
		0.0–2.5	2.5–5.0	5.0–10.0	10.0–15.0	15.0–20.0	20.0–25.0	25.0–30.0	30.0–35.0	Mean
Normal	No. of subjects	20	26	0	0	0	0	0	0	2.66
	% of subjects	43.5	66.5	0	0	0	0	0	0	
With hepatitis	No. of patients	0	1	16	19	17	18	8	4	17.03
	% of patients	0	1.2	19.3	22.9	20.5	21.7	9.6	4.8	

have been observed with sera from patients with other types of hepatic parenchymal damage, such as that resulting from toxic hepatitis and cirrhosis of the liver. With the modified technique the normal range in units of turbidity is nearly identical with that observed by Maclagan (2) and the degree of increase in turbidity occurring in the presence of liver damage is comparable with results obtained with the original technique.

SUMMARY

A modification of the thymol turbidity reaction has been described which permits turbidimetric measurement in the spectrophotometer. The method has been simplified, and more exact standardization of the procedure has been achieved by the use of a barium sulfate standard. Determination of the thymol turbidity reaction of sera from forty-six normal subjects gave values with a range of from 0 to 4.7 units, with a mean of 2.66 units. In 83 patients ˙with infectious hepatitis the thymol turbidity was increased in 82 and the mean for the group of patients was 17.03 units.

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STUDIES ON PHOSPHATASE-CONTAINING ENZYME PRODUCTS FOR HYDROLYSIS OF COCARBOXYLASE IN BREWERS' TYPE YEASTS AND WHEAT EMBRYO AT VARIOUS HYDROGEN ION CONCENTRATIONS*

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While the author was engaged in a recent study on the availability of thiamine in brewers' type yeasts (1), attempts were made to reduce to the minimum the errors inherent in the thiochrome method of determining vitamin B_1 in foods, as developed by Hennessy and Cerecedo (2) and modified by Conner and Straub (3) and the research staff of Merck and Company (4). It was found that lack of optimum hydrogen ion concentration before incubation with taka-diastase produced enormous errors in the vitamin B_1 content of dried yeasts. Such results stimulated the extension of our study to other foods and food products.

For the enzymatic hydrolysis of cocarboxylase in foods Hennessy and Cerecedo (2) in their original method adjusted their vitamin solutions before incubation to pH 6.5 to 7.0 when they used a kidney extract as a source of phosphatase. They incubated their samples at 37° for 3 hours. Employing commercial phosphatase preparations, Conner and Straub (3) found the optimum pH before incubation to be 4.5 to 4.7. The latter investigators used a temperature of 45° for a period of 2 hours. We concur with the observations of Conner and Straub with regard to the optimum pH for the enzymatic hydrolysis of cocarboxylase in foods, which can be obtained by the use of 6 to 7 cc. of 2 N sodium acetate solution. We, however, followed the procedure of Brown, Hamm, and Harrison (5) of using a temperature of 52° for incubation for 2 hours, as suggested by Andrews (6).

In this study we were impressed with the high incidence of hydrolysis of the cocarboxylase in commercial wheat embryo at extremely low hydrogen ion concentrations compared with the hydrolysis of combined thiamine in two brands of brewers' type of dried yeasts.[1] It was also thought of

[1] Kindly furnished by Standard Brands Incorporated, New York.

interest to determine the efficiency of enzymatic hydrolysis of cocarboxylase in the yeasts and wheat embryo as influenced by the source of the phosphatase. Accordingly, four commercial products were studied that are used extensively in the determination of thiamine in foods and food products; namely, taka-diastase,[2] clarase,[3] mylase P,[4] and polidase.[5] The results of this investigation are submitted in Tables I to VIII.

It will be noted from Table I that at pH 2.5 to 2.65 neither taka-diastase, clarase, nor polidase hydrolyzed any cocarboxylase in brewers' type Yeast 1, which had a total thiamine content of 754.5 γ per gm. On the other hand, great efficiency of hydrolysis of cocarboxylase of wheat embryo was effected by all these commercial phosphatase preparations at even more acid hydrogen ion concentrations; i.e., taka-diastase, at pH 1.92, 75.2 per cent; clarase, at pH 2.26, 81.8 per cent; polidase, at pH 2.11, 87.6 per cent; and mylase, at pH 1.99, 77.7 per cent (Table III). In brewers' type Yeast 2, at pH about 3 we still have low yields of hydrolysis of combined thiamine: taka-diastase, at pH 3.04, 11.0 per cent; clarase, at pH 3.22, 12.9 per cent; polidase, at pH 3.15, and even at 3.97, only 1.4 per cent; and mylase, at pH 2.95, 36.2 per cent (Table II). However, at pH 2.97 mylase hydrolyzed 92.5 per cent and at pH 3.08 polidase hydrolyzed 90.1 per cent of the cocarboxylase of wheat embryo (Table III). At the low hydrogen ion concentrations large proportions of cocarboxylase were also hydrolyzed in commercial wheat embryo previously extracted with organic solvents (viobin)[6] (Table V) but not to the same extent as in unextracted commercial wheat germ (Table III).

When 5 cc. of a 10 per cent solution of the various phosphatase products were used, which is equivalent to 0.5 gm. of dry substance, taka-diastase, clarase, and mylase proved equally efficient in the case of brewers' type Yeast 1 and polidase slightly inferior. However, on brewers' type Yeast 2, polidase was definitely the poorest source of phosphatase, since the maximum efficiency of hydrolysis was only 48.5 per cent. It would appear then that in the evaluation of the efficiency of commercial sources of phosphatases for the hydrolysis of combined thiamine in foods one must consider the substrate as well as the source of the enzyme. When, however, used in high concentration (Table III) polidase was just as efficient as taka-diastase, clarase, and mylase in hydrolyzing the cocarboxylase of wheat embryo. Of course, wheat embryo was found to have only 12 γ of

[2] Parke, Davis and Company, Detroit, Michigan.
[3] Takamine Laboratories, Inc., Clifton, New Jersey.
[4] Wallerstein Laboratories, New York 16, New York.
[5] Schwarz Laboratories, Inc., New York.
[6] Kindly furnished by The Viobin Corporation, Monticello, Illinois.

cocarboxylase compared with 483 and 317 γ of combined thiamine in the yeasts and it is possible polidase has sufficient phosphatase for hydrolyzing the combined thiamine in the wheat germ but not enough for the yeasts.

TABLE I

Influence of pH and Source of Phosphatase on Thiamine Content of Brewers' Type Yeast 1

Source of phosphatase*	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
		γ per gm.		γ per gm.	
Taka-diastase	2.50	263.6	34.9	0	0
	2.73	281.8	37.3	10.2	2.1
	3.45	563.6	74.7	292.0	60.5
	3.92	568.3	75.3	296.7	63.5
	4.42	627.3	83.2	355.7	73.7
	4.65	718.2	95.2	446.6	92.5
	4.70	754.5	100.0	482.9	100.0
	4.89	718.2	95.2	446.6	92.5
Clarase	2.56	272.3	36.0	0.7	0.2
	2.91	281.8	37.3	4.2	0.9
	3.51	490.9	66.5	219.3	45.5
	3.89	636.3	84.3	364.7	75.5
	4.38	700.0	92.8	428.4	88.7
	4.58	681.8	90.5	410.2	84.9
	4.70	754.5	100.0	482.9	100.0
Polidase	2.65	263.6	34.9	0	0
	3.51	281.8	37.3	10.2	2.1
	4.00	336.3	43.2	64.7	13.4
	4.42	536.3	71.1	264.7	52.8
	4.63	663.6	88.5	392.0	81.1
	4.91	681.8	90.4	410.2	85.3
	5.18	681.8	90.4	410.2	85.3
Mylase	2.71	281.8	37.3	10.2	2.1
	3.28	590.9	78.4	319.3	66.1
	3.90	609.1	80.8	337.5	70.0
	4.30	754.5	100.0	482.9	100.0
	4.53	754.5	100.0	482.9	100.0
	4.73	736.3	97.6	464.7	96.2
	4.82	736.3	97.6	464.7	96.2
	5.15	700.0	92.8	428.4	88.7

* 5 cc. of a 10 per cent solution.

On the other hand, Clausen and Brown (7) found polidase to be the most potent commercial phosphatase for bread which is a low cocarboxylase-containing product. Their results were not confirmed, however, by Brown, Hamm, and Harrison (5).

In order to determine further the relative efficiency of the commercial phosphatase products, they were studied in different concentrations on the yeasts and wheat embryo. From Tables VI and VII it is apparent that when 5 cc. of a 10 per cent solution are employed, equivalent to 0.5 gm. of dry substance, taka-diastase, clarase, and mylase are equally efficient and that polidase is definitely poorer. At the 1 per cent concentration, equivalent to 0.05 gm. of dry substance, polidase is a complete failure, there being no difference among the other three phosphatase preparations.

TABLE II

Influence of pH and Source of Phosphatase on Thiamine Content of Brewers' Type Yeast 2

Source of phosphatase[*]	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
		γ per gm.		γ per gm.	
Taka-diastase	3.04	206.2	42.2	35.3	11.0
	3.73	383.1	78.5	212.2	66.9
	4.02	432.5	88.7	261.6	82.7
	4.38	488.3	100.0	317.4	100.0
	4.48	487.0	99.8	316.1	99.6
Clarase	3.22	211.7	43.5	40.8	12.9
	3.97	357.7	73.6	186.8	58.9
	4.21	396.7	80.1	225.8	71.2
	4.53	435.1	89.2	264.2	83.9
	4.70	454.5	93.1	283.6	89.4
Polidase	3.15	175.3	35.8	4.4	1.4
	3.97	175.3	37.1	4.4	1.4
	4.28	211.7	43.4	40.8	12.9
	4.52	227.3	46.5	76.4	24.1
	4.85	324.8	66.6	153.9	48.5
Mylase	2.95	285.7	58.5	114.8	36.2
	3.79	415.7	85.2	244.8	77.2
	4.10	415.7	85.2	244.8	77.2
	4.45	454.5	93.1	283.6	89.4
	4.68	441.5	91.9	270.6	85.3

[*] 5 cc. of a 10 per cent solution.

However, at the 0.1 per cent concentration, equivalent to 0.005 gm. of dry substance, clarase is poorer than either taka-diastase or mylase, the latter two being about equally efficient. With wheat embryo (Table IV) the striking difference is apparent only in the greatest dilution, equivalent to 0.001 gm. of dry substance. At that dilute concentration, mylase

proved the most efficient, yielding 56 per cent of hydrolyzed cocarboxylase compared with only 19.7 per cent of combined thiamine hydrolyzed by either taka-diastase, clarase, or polidase.

TABLE III

Influence of pH and Source of Phosphatase on Thiamine Content of Commercial Wheat Embryo

Source of phosphatase*	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
		γ per gm.		γ per gm.	
Taka-diastase	1.73	18.7	82.6	7.5	62.0
	1.92	20.3	87.0	9.1	75.2
	2.21	20.3	87.0	9.1	75.2
	3.07	21.8	93.6	10.6	87.6
	4.60	23.3	100.0	12.1	100.0
	4.88	22.1	94.3	10.9	90.1
	5.13	22.1	94.3	10.9	90.1
Clarase	1.97	20.0	85.8	8.8	72.9
	2.26	21.1	90.6	9.9	81.8
	2.51	21.1	90.6	9.9	81.8
	3.15	21.1	90.6	9.9	81.8
	4.87	23.3	100.0	12.1	100.0
	5.05	23.3	100.0	12.1	100.0
Polidase	2.03	20.9	89.7	9.7	80.2
	2.11	21.8	93.6	10.6	87.6
	3.08	22.1	94.3	10.9	90.1
	4.13	23.3	100.0	12.1	100.0
	4.73	23.3	100.0	12.1	100.0
	4.97	23.3	100.0	12.1	100.0
Mylase	1.99	20.6	88.3	9.4	77.7
	2.38	21.1	90.6	9.9	81.8
	2.97	22.4	96.1	11.2	92.5
	4.20	21.9	94.0	10.7	88.2
	4.69	23.3	100.0	12.1	100.0
	4.93	23.3	100.0	12.1	100.0
	5.22	22.7	97.3	11.5	95.0

* 5 cc. of a 10 per cent solution.

Another point of interest is our observation that the phosphatase in taka-diastase and mylase for the hydrolysis of cocarboxylase in brewers' type Yeast 2 is easily destroyed by a few minutes contact at a low pH within a narrow range of hydrogen ion concentration (Table VIII). The yeast extracts containing 295.5 γ of combined thiamine were allowed 3 minutes

contact at pH levels ranging from 1.7 to 2.32 and were then immediately buffered to pH 4.52 to 4.65, at which hydrogen ion concentration optimum results were obtained. With taka-diastase, a change from pH 1.7 to 2.04 was accompanied by a change from 0 to 82.4 per cent hydrolyzed cocarboxylase and at pH 2.26 a 100 per cent yield was obtained. With mylase, a change from pH 1.75 to 2.05 resulted in an increase of 42.3 per cent hydrolysis of cocarboxylase and at pH 2.32 the phosphatase was completely stable during 3 minutes contact. In other words, by 3 minutes contact at

TABLE IV

Influence of Source and Concentration of Phosphatase on Thiamine Content of Commercial Wheat Embryo

Source of phosphatase	Concentration of phosphatase		pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
	per cent	*cc.*		*γ per gm.*		*γ per gm.*	
Taka-diastase	10.0	5	4.53	22.4	100.0	11.6	100.0
	1.0	5	4.53	20.9	93.3	10.1	90.4
	0.1	5	4.53	18.7	83.7	7.9	68.4
	0.1	1	4.53	13.1	58.5	2.3	19.7
Clarase	10.0	5	4.65	22.4	100.0	11.6	100.0
	1.0	5	4.65	18.2	81.2	7.4	62.9
	0.1	5	4.65	16.3	72.8	5.5	47.3
	0.1	1	4.65	13.1	58.5	2.3	19.7
Polidase	10.0	5	4.70	22.4	100.0	11.6	100.0
	1.0	5	4.70	22.4	100.0	11.6	100.0
	0.1	5	4.70	18.2	81.2	7.4	62.9
	0.1	1	4.70	13.1	58.5	2.3	19.7
ylase	10.0	5	4.69	22.4	100.0	11.6	100.0
	1.0	5	4.69	22.4	100.0	11.6	100.0
	0.1	5	4.69	18.2	81.2	7.4	62.9
	0.1	1	4.69	17.3	77.2	6.5	56.0

pH 1.7 to 1.75 the cocarboxylase phosphatase in taka-diastase and mylase is entirely destroyed but contact for the same period at pH 2.26 to 2.32 has not produced any deleterious effect on this enzyme in either of these commercial preparations.

The fact that the cocarboxylase in wheat embryo can be hydrolyzed by phosphatases at a much lower hydrogen ion concentration than the combined thiamine of brewers' type yeasts would suggest that thiamine exists in a differently combined form in wheat germ than in the type of yeasts studied.

TABLE V

Influence of pH and Source of Phosphatase on Thiamine Content of Commercial Solvent-Extracted Wheat Embryo (Viobin)

Source of phosphatase*	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
		γ per gm.		γ per gm.	
Taka-diastase	1.97	14.3	61.3	0	0
	2.30	17.3	74.2	2.9	32.7
	3.48	18.7	80.3	4.3	48.9
	4.73	23.3	100.0	8.8	100.0
	4.91	23.3	100.0	8.8	100.0
Clarase	2.17	21.2	90.6	6.8	77.3
	2.57	22.1	94.3	7.7	87.5
	2.98	22.1	94.3	7.7	87.5
	4.51	23.3	100.0	8.8	100.0
	4.97	23.3	100.0	8.8	100.0
Polidase	2.15	12.7	54.5	0	0
	2.43	14.2	60.7	0	0
	3.04	20.3	87.0	5.9	67.0
	4.27	21.5	92.2	7.1	80.7
	4.69	23.3	100.0	8.8	100.0
Mylase	1.94	13.9	59.2	0	0
	2.30	17.3	74.2	2.9	32.9
	3.38	19.7	84.3	5.3	60.2
	4.23	21.5	92.3	7.1	80.7
	4.77	23.3	100.0	8.8	100.0

* 5 cc. of a 10 per cent solution.

TABLE VI

Influence of Source and Concentration of Phosphatase on Thiamine Content of Brewers' Type Yeast 1

Source of phosphatase	Concentration of phosphatase (5 cc.)	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
	per cent		γ per gm.		γ per gm.	
Taka-diastase	10.0	4.75	746.0	100.0	477.4	100.0
Clarase	10.0	4.70	727.3	97.5	458.7	96.1
Polidase	10.0	4.71	666.7	89.4	398.1	81.3
Mylase	10.0	4.75	746.0	100.0	477.4	100.0
Taka-diastase	1.0	4.57	691.0	92.6	422.4	88.8
Clarase	1.0	4.58	691.0	92.0	422.4	88.8
Polidase	1.0	4.68	291.0	39.0	22.4	4.7
Mylase	1.0	4.70	691.0	92.6	422.4	88.8
Taka-diastase	0.1	4.75	624.0	83.6	355.4	74.4
Clarase	0.1	4.71	309.1	40.4	40.5	8.4
Mylase	0.1	4.68	624.0	83.6	355.4	74.4

TABLE VII

Influence of Source and Concentration of Phosphatase on Thiamine Content of Brewers'
Type Yeast 2

Source of phosphatase	Concentration of phosphatase (5 cc.)	pH	Thiamine content	Per cent of maximum yield	Combined thiamine	Per cent of combined thiamine hydrolyzed
	per cent		γ *per gm.*		γ *per gm.*	
Taka-diastase...........	10.0	4.65	454.5	100.0	295.4	100.0
Clarase.................	10.0	4.71	448.0	98.7	288.9	97.8
Polidase................	10.0	4.69	357.1	78.5	197.9	66.9
Mylase.................	10.0	4.62	454.5	100.0	295.4	100.0
Taka-diastase...........	1.0	4.53	402.6	88.6	243.5	82.4
Clarase.................	1.0	4.60	402.6	88.6	243.5	82.4
Polidase................	1.0	4.59	188.3	41.4	29.2	9.9
Mylase.................	1.0	4.62	402.6	88.6	243.5	82.4
Taka-diastase...........	0.1	4.70	388.7	85.6	229.6	77.7
Clarase.................	0.1	4.72	318.5	70.2	159.4	53.9
Mylase.................	0.1	4.67	402.6	88.6	243.5	82.4

TABLE VIII

Influence of 3 Minutes Contact of Taka-diastase and Mylase in Slightly Acid Solutions
on Their Efficiency to Hydrolyze Cocarboxylase in Brewers' Type Yeast 2

Source of phosphatase	Initial pH	pH after 3 min. contact and after buffering	Thiamine content	Combined thiamine	Per cent of combined thiamine hydrolyzed
			γ *per gm.*	γ *per gm.*	
Taka-diastase		4.58	454.5	295.5	100.0
	1.70	4.55	159.0	0	0
	1.88	4.55	194.8	35.8	12.1
	2.04	4.56	402.6	243.6	82.4
	2.26	4.57	454.5	295.5	100.0
Mylase		4.55	454.5	295.5	100.0
	1.75	4.52	246.8	87.8	29.8
	1.86	4.53	272.7	113.7	38.4
	1.93	4.55	292.2	133.2	45.1
	2.05	4.52	374.0	215.0	72.1
	2.12	4.55	374.0	215.0	72.1
	2.27	4.62	406.5	247.5	83.6
	2.32	4.65	454.5	295.5	100.0

SUMMARY

At low hydrogen ion concentrations of 2.5 to 2.7, practically no free thiamine is liberated by commercial phosphatase preparations from the cocarboxylase of two brewers' type yeasts. On the other hand, at the same range of pH, 75 to 88 per cent of combined thiamine is hydrolyzed

by such phosphatases from commercial wheat embryo. These results suggest that cocarboxylase may exist in different combinations in these food products.

A study was made of the relative efficiency of four commercial phosphatase preparations for the hydrolysis of cocarboxylase in two brewers' type yeasts and in commercial wheat embryo. For the yeasts, polidase proved the poorest. In very dilute concentrations, taka-diastase and mylase were the most efficient. However, in higher concentrations, taka-diastase, clarase, and mylase proved of equal value. For wheat embryo, when used in amounts equivalent to 0.5 gm. of dry substance, all the four commercial phosphatase preparations were of equal efficiency.

After 3 minutes contact at pH 1.7 to 1.75, the phosphatases in taka-diastase and mylase lost completely their activity to hydrolyze the cocarboxylase in a brewers' type of yeast. However, on contact during the same period at pH 2.26 and 2.32, the phosphatases in taka-diastase and mylase respectively were found completely stable.

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EFFECT OF INHIBITION OF GLYCOLYSIS AND COMPOUNDS RELATED TO GLYCOLYSIS ON ACETYLCHOLINE SYNTHESIS*

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During prolonged activity of cholinergic systems an acetylcholine-like substance is synthesized locally (1, 2) since more acetylcholine can be released during stimulation than is present in the non-stimulated organ. The energy necessary to perform various physicochemical processes is usually supplied through oxidative processes and glycolysis. It has already been found that the addition of glucose (3–6) and some of the C_4 dicarboxylic acids (7) in relatively low concentrations increases the synthesis of acetylcholine *in vitro*. The purpose of the following investigation was to determine (1) the amount of acetylcholine synthesized by minced fresh brain after severe inhibition of its glycolysis and respiration, and (2) the effect of added amounts of various intermediary products of glycolysis on the synthesis of acetylcholine.

Method

The effect of the substances on the acetylcholine synthesis was studied by the method described previously (8). Mixtures containing varying amounts of the substances used (pH corrected to 7.4), 100 mg. of minced fresh frog brain, 3 mg. of physostigmine salicylate, and 3 cc. of Ringer's solution were shaken and incubated aerobically, if not otherwise indicated, for 4 hours at 37°. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog. It was also ascertained whether the substances modified the sensitivity of the rectus abdominis muscle to the acetylcholine content of the mixtures during the 2 minutes of immersion for the biological assay by adding the substances to incubated control mixtures after incubation. If the substances modified the sensitivity of the rectus abdominis muscle to acetylcholine, the changes were taken into account by the calculation.

Calculation—The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical non-incubated mixtures. The amount of acetylcholine synthesized in the control mixtures containing only brain,

physostigmine, and Ringer's solution was taken as 100 per cent. The acetylcholine content of the mixtures containing the various substances used was expressed as a per cent of the control.

Results

Effect of Inhibition of Glycolysis and Respiration on Acetylcholine Synthesis—Quastel, Tennenbaum, and Wheatley (3) found that fresh minced brain *in vitro* synthesized much more acetylcholine aerobically than anaerobically. However, in the presence of adenosine triphosphate a considerable amount of acetylcholine is synthesized anaerobically (9, 10).

TABLE I

Effect of Inhibitors of Glycolysis on Anaerobic Synthesis of Acetylcholine

Substance	Amount of acetylcholine synthesized in per cent of control*			
	Amounts of substances added to 100 mg. frog brain			
	3 mg.	0.3 mg.	0.03 mg.	0.003 mg.
Fluoride....................	109	90	82	81
Monoiodoacetate..............	19	20	43	103

* Each value represents the average of eight separate experiments. The standard error of the mean for each value was less than ±5 per cent. The amount of acetylcholine synthesized in micrograms per 100 mg. of frog brain, followed by the standard error of the mean, was 0.32 ± 0.015.

With the preparation and technique described above an average of 0.32 γ of acetylcholine was synthesized anaerobically (without addition of adenosine triphosphate) and 0.95 γ aerobically. It would appear, therefore, that most of the energy required for the synthesis of acetylcholine is supplied by oxidative processes if oxygen is available and by anaerobic glycolysis in the absence of oxygen when adenosine triphosphate is available.

To test the effect of inhibition of respiration and anaerobic glycolysis on the amount of acetylcholine synthesized, known inhibitors of the glycolysis (monoiodoacetate, fluoride (11, 12)) were added to the mixtures before incubation and the mixtures were incubated in nitrogen instead of oxygen.

The amounts of acetylcholine synthesized are given in Table I. Fluoride did not significantly modify the amount of acetylcholine synthesized. Since fluoride is known to inhibit the transformation of phosphoglycerate to phosphopyruvate and to inhibit various phosphorylations, it is likely that the acetylcholine synthesis is not modified by the products of phosphoglycerate and does not depend on fluoride-sensitive phosphorylations.

Monoiodoacetate decreased the amount of acetylcholine synthesized, probably because of the inactivation of the —SH group of the enzyme involved in the acetylcholine synthesis rather than the inhibition of the transformation of triose phosphate to 3-phosphoglycerate.

In the following series of experiments inhibitors of glycolysis (monoiodoacetate, glyceraldehyde, fluoride) were added to the mixtures before aerobic incubation.

The amounts of acetylcholine synthesized are given in Table II. The results suggest that inhibition of glycolysis does not inhibit acetylcholine synthesis significantly. Glyceraldehyde in concentrations of 0.0055 M is known to decrease the glycolysis in brain by 90 per cent (11, 12), but, in concentrations as high as 0.01 M, glyceraldehyde decreased the acetylcholine synthesis only by 30 per cent. Fluoride in concentrations of 0.01 M is

TABLE II

Effect of Inhibitors of Glycolysis on Synthesis of Acetylcholine

Substance	Amount of acetylcholine synthesized in per cent of control*					
	Amounts of substances added to 100 mg. frog brain					
	30 mg.	3 mg.	0.3 mg.	0.03 mg.	0.003 mg.	0.0003 mg.
Glyceraldehyde.........		70	94	102	100	99
Fluoride...............	42	77	96	105	105	104
Monoiodoacetate.......		21	47	78	102	104

* Each value represents the average of eight separate experiments. The standard error of the mean for each value was less than ±5 per cent. The amount of acetylcholine synthesized in micrograms per 100 mg. of frog brain, followed by the standard error of the mean, was 0.95 ± 0.033.

known to inhibit the transformation of phosphoglycerate to phosphopyruvate by 100 per cent (11, 12), but, in concentrations as high as 0.024 M, it inhibited the acetylcholine synthesis only by 23 per cent and in concentrations of 0.24 M by 58 per cent. Monoiodoacetate in concentrations of 0.000054 M is known to inhibit the transformation of triose phosphate to 3-phosphoglycerate by 97 per cent (11, 12), but, in concentrations as high as 0.00054 M, it inhibited the acetylcholine synthesis only by 53 per cent and in concentrations of 0.0054 M by 80 per cent. The inhibition of acetylcholine synthesis by monoiodoacetate was probably due to inactivation of the —SH groups of the enzymes involved in the synthesis. Relatively large concentrations of monoiodoacetate were required for complete inhibition of the acetylcholine synthesis, probably because the present experiments were carried out at pH 7.4, a pH unfavorable for the action of monoiodoacetate (13).

Effect of Intermediary Products of Carbohydrate Metabolism on Acetylcholine Synthesis—The amounts of acetylcholine synthesized in the presence of some intermediary products of carbohydrate metabolism, some organic phosphates, and their decomposition products are given in Table III.

TABLE III

Effect of Various Substances on Acetylcholine Synthesis

Substance	Amount of acetylcholine synthesized in per cent of control*					
	Amounts of substances added to 100 mg. frog brain					
	30 mg.	3 mg.	0.3 mg.	0.03 mg.	0.003 mg.	0.0003 mg.
Fructose diphosphate..........		220	184	143	99	98
Dihydroxyacetone monophosphate.......................		57	77	118	123	112
β-Glycerophosphate............		103	105	105	102	104
Pyruvic acid...................	114	116	110	104	104	99
Acetyl phosphate..............		62	88	100	104	
Lactic acid....................	142	137	124	120	111	103
Acetic "		102	101	104	102	102
Acetoacetic acid...............		105	101	103	100	98
Acetone......................		96	110	104	97	102
Acetaldehyde.................		20	49	75	88	92
Ethyl alcohol........`........		91	105	100	99	97
Glycerol.....................		101	98	100	103	100
Adenosine triphosphate........		177	157	137	113	102
Creatine phosphate............		147	134	118	112	108
Creatine.....................		121	116	108	104	103
Creatinine....................		103	97	101	98	100
Inosinic acid (muscle).........				70	72	76
Ammonia.....................	15	34	67	90	101	102
Pyrophosphate	80	98	112	117	111	104

* Each value represents the average of eight separate experiments. The standard error of the mean for each value was less than ±5 per cent. The amount of acetylcholine synthesized in micrograms per 100 mg. of frog brain, followed by the standard error of the mean, was 0.95 ± 0.033.

Fructose diphosphate, dihydroxyacetone monophosphate (low concentrations), pyruvic acid, and lactic acid increased the amount of acetylcholine synthesized. The increase of synthesis was least with pyruvic acid. The end-products of glycolysis tested (acetic acid, acetoacetate, acetone, ethyl alchol) did not modify the synthesis.

Adenosine triphosphate, a compound capable of receiving, storing, and transmitting to cellular mechanisms (9, 10, 14–16) the energy produced by glycolytic and some other processes, increased the amount of acetylcholine synthesized. A smaller increase was found in the presence of creatine phosphate, a reservoir for the phosphate group used .during the

esterification of carbohydrates, and creatine. Creatinine did not modify the synthesis. Decomposition products of adenosine triphosphate (ammonia, inosinic acid (17)) decreased the synthesis.

Pyrophosphate in low concentrations increased the synthesis and decreased it in higher ones. It is known that pyrophosphate in low concentrations (18, 19) protects the —SH group from oxidation and in higher concentrations inhibits various enzyme systems (20–22).

Comment

Intermediary products of glycolysis and organic phosphates may increase the synthesis of acetylcholine, suggesting that this increase of acetylcholine synthesis is due to energy derived from glycolysis. Furthermore, the amount of acetylcholine synthesized may be regulated by the dynamic equilibrium of potentiator and inhibitor (ammonia, inosinic acid, etc.) substances.

The results also suggest that if oxygen is available most of the energy required for the synthesis of acetylcholine is supplied by oxidative processes, and, in the absence of oxygen, by anaerobic glycolysis. However, it appears that some acetylcholine synthesis can occur even in the absence of glycolysis and free oxygen supply.

SUMMARY

1. The effect of some products of glycolysis, some substances involved in the esterification of carbohydrates, and some inhibitor substances of glycolysis on the synthesis of acetylcholine was investigated.

2. The synthesis was increased in the presence of fructose diphosphate, dihydroxyacetone monophosphate (low concentrations), pyruvic acid, lactic acid, adenosine triphosphate, creatine phosphate, creatine, and pyrophosphate (low concentrations).

3. The synthesis was not modified in the presence of β-glycerophosphate, acetic acid, acetoacetic acid, acetone, glycerol, ethyl alcohol, and creatinine.

4. The synthesis was decreased in the presence of dihydroxyacetone monophosphate (higher concentrations), acetyl phosphate (higher concentrations), acetaldehyde, ammonia, inosinic acid, pyrophosphate (higher concentrations), glyceraldehyde, fluoride, and monoiodoacetate.

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THE EFFECT OF THE PRECEDING DIET
UPON FASTING KETONEMIA

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Earlier investigations (1) upon the metabolism of animals with fatty livers suggested that further information on this subject might be obtained by a study of ketosis in these animals. It was observed that conflicting evidence had been presented in regard to the influence of the constituents of the preceding diet upon the degree of fasting ketosis. Deuel and Hallman (2) found an appreciable increase in fasting ketonuria following the ingestion of a high fat, low protein diet for only 1 day in comparison with the level in the rats previously on a stock diet. This finding does not seem to be wholly in accord with the conclusions of MacKay *et al.* (3) that the degree of fasting ketosis is related to the amount of stored protein available for catabolism during fasting and that the protein intake preceding the fast is the principal determinant of the stored protein. If the degree of fasting ketosis is related to the stores of body protein, it seems unlikely that these stores should be changed sufficiently after 1 day on a low protein diet to have such a profound effect upon the fat metabolism during the ensuing period. A ketonuria would be associated with an appreciable ketonemia, since the renal threshold for the ketone bodies in the rat has been shown to be 25 to 30 mg. per cent (4).

Later MacKay and coworkers (5) found the amount of fat in the preceding diet to have no significant influence upon the following fasting ketosis and the factor determining the extent of fasting ketosis to be the amount of antiketogenic material from the protein catabolized. Roberts and Samuels (6) have presented evidence which suggests that the degree of fasting ketosis is influenced by the fat content of the preceding diet. However, a very high intake of fat (71 per cent of the diet) was required to produce a small elevation of ketone bodies in the blood and urine.

The present study is the first of a series of studies on ketosis initiated in 1943. It was designed to evaluate the influence of some constituents of the preceding diet upon the following fasting ketonemia. The experimental diets preceding the fasting periods varied widely in protein, fat, and carbohydrate content. A micromethod was developed for the determination of ketone bodies in 0.2 cc. of blood. With this method the changes in the blood ketones could be followed in individual animals throughout the fasting periods. Thus it allowed the accumulation of more

extensive data than have been obtained previously. Due to marked individual variations, this procedure should give more reliable data on ketonemia than those obtained by sacrificing a small number of animals (two or three usually) at the end of each time period. Most of the data in the literature have been obtained by the latter method.

The protein intake preceding the fasting period was found to be more directly related to the degree of fasting ketonemia than were the intakes of the other constituents examined. However, the effect of ingested protein seemed to be much greater than could be explained by the protein stores made available as antiketogenic material during fasting.

TABLE I

Composition of Diets

All diets were supplemented daily with 1 dried yeast tablet (400 mg.) and 2 drops of cod liver oil. To each 100 gm. of Diets 2 and 5 were added 1.0 and 0.5 gm. of choline and cystine respectively.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Casein...................	5.0	5.0	20.0	20.0	20.0	30.0
Starch...................	24.0	36.5	24.0	36.5	16.5	0.0
Glucose*...............	24.0	36.5	24.0	36.5	16.5	0.0
Cellu flour.............	2.0	2.0	2.0	2.0	2.0	2.0
Salt mixture†..........	5.0	5.0	5.0	5.0	5.0	5.0
Lard...................	40.0	15.0	25.0	0.0	40.0	63.0

* Generously supplied by the Corn Products Refining Company, New York.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

EXPERIMENTAL

White male rats weighing approximately 200 gm. (Group II) and 150 gm. (Group III) were used. The former animals were secured from Sprague-Dawley, Inc., and the latter ones from the Albino Farms. For 2 weeks after arrival in the laboratory, the animals received Purina dog chow and distilled water. The animals were then fasted for 48 hours and determinations made of the fasting blood ketone levels to serve as control values. After a return to the stock diet for 8 days, all the animals received the experimental diets and distilled water *ad libitum* for 23 days. The composition of the diets is given in Table I. The daily food intake and weight changes were recorded. To determine more accurately the start of the fasting period, the rats were always fed at 3 p.m. each day and the food cups removed 23 hours later. The cups were not returned to the cages on the day the fasting period began and 3 p.m. was considered the start of the fast. Total blood acetone bodies were determined on blood

samples collected at intervals of 12, 24, and 48 hours after the start of the fast. The control and 12 hour fasting values were not determined on the animals of Group III. The room temperature was maintained between the limits of 23–27°.

Methods

Total blood acetone bodies were determined by a combination and modification of the methods of Shipley and Long (4) and Block and Bolling (7). The former method was followed in detail except for the following modifications. A 0.2 cc. sample of blood was withdrawn directly from the clipped tail into an oxalated blood pipette and the tungstic acid filtrate prepared as suggested. The acetone was distilled from 4 cc. of filtrate to which had been added 12 cc. of water. This permitted duplicate determinations on one sample of blood and the collection of 9 cc. of distillate desired for colorimetric determinations. The oxidation of the ketone bodies to acetone and the distillation were completed as detailed in the original method. Ice water was circulated through the condenser to prevent loss of acetone. Large test-tubes, containing 1 cc. of 2 per cent sodium bisulfite, served as receivers during the distillation and later as absorption tubes in the Evelyn photoelectric colorimeter. After the distillation, their contents were made up to a volume of exactly 10 cc. and 10 cc. of 40 per cent sodium hydroxide added. The amount of acetone in the distillate was then determined according to the colorimetric method of Block and Bolling. Calibration curves were prepared from acetone standards run through the entire procedure. This standardization corrects for the error that would be introduced by the known loss of acetone during the distillation. As an additional precaution, at least three points on the calibration curve were checked by running acetone standards concurrently with each series of blood ketone estimations. The deviations of these from the standard curve and of serial determinations on the same blood filtrate were less than 5 per cent. Thus this micromethod was found reliable and it permitted the desired serial determinations of the blood ketone bodies, measured as acetone, on the same animal.

Apparent differences in the results of this study were analyzed for significance by the t method of Fisher (8) and only those showing a P value of less than 0.01 were considered significant. Included with the data are the standard errors of the means calculated as $\sqrt{\Sigma d^2/N-1}/\sqrt{N}$.

Results

The control fasting ketonemias of the groups selected on the basis of body weights were found quite uniform (average value of 12 ± 1 mg. per cent). Thus the groups were distributed on the diets without regard to

the blood ketone levels, since they were similar. The degree of ketonemia at the end of various periods of fasting following the experimental diets is summarized in Table II. Similar blood ketone levels were present at the end of the various fasting periods in the animals on the low protein diets, Nos. 1 and 2. The same was true of the animals on the higher protein diets, Nos. 3 to 6. A marked increase in the blood ketone bodies occurred at the end of the 24 and 48 hour fast in the animals on Diets 1 and 2 as compared with those on the other diets. In Table III, the protein and fat intakes of both Groups II and III are calculated on the basis of body weight in order to give a better comparison of the dietary effects, since the amount of food ingested varies considerably with marked changes

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TABLE II

Fasting Ketonemia after Special Diets

</div>

The animals received the diets for 23 days. There were eight animals weighing approximately 200 gm. on all diets except Diet 6 which included ten animals, 150 gm. in weight. Blood ketone levels were determined at the end of various periods that began with the removal of the food cups, 24 hours after the last feeding.

Group and diet No.	Food intake per day	Change in weight	Blood ketone bodies during fasting		
			12 hrs.	24 hrs.	48 hrs.
	gm.	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
II-1	9.8 ± 0.1	1.1 ± 1.8	8 ± 1	20 ± 3	44 ± 3
II-2	12.3 ± 0.5	5.2 ± 2.2	7 ± 0	22 ± 2	38 ± 2
II-3	12.1 ± 0.7	24.0 ± 3.3	8 ± 1	9 ± 1	15 ± 1
II-4	17.5 ± 0.6	27.2 ± 2.4	7 ± 0	10 ± 1	14 ± 2
II-5	10.7 ± 0.2	30.9 ± 4.7	6 ± 1	7 ± 0	13 ± 2
III-6	7.0 ± 0.3	34.3 ± 3.8		7 ± 1	13 ± 2

in the fat content of the diet. The preceding protein intakes were found to be related to the fasting blood ketone levels.

Effect of Fat Intake in Preceding Diet—To determine whether or not this is a factor governing fasting ketosis, the rats were fed fat at levels ranging from traces to 63 per cent of the diet (Table II). If comparisons of results after various dietary fat levels are made on animals ingesting the diets with the same protein content (either 5 or 20 per cent), the ketonemias were not significantly different after the various fasting periods. For example, the ketone levels were similar after the 5 per cent protein diets, one of which contained 15 per cent and the other 40 per cent fat. The same is true when the diets contained 20 per cent protein and from traces to 40 per cent fat. Also there were no appreciable differences in the fasting ketonemia after marked changes in the fat intake if the absolute amount of protein ingested was approximately the same (Table III). The animals in Groups II-3,

III-6, and III-2 ingested 1.13 to 1.17 gm. of protein per 100 gm. of body weight per day during the experimental period and received from 38 to 83 per cent of their calories as fat. Nevertheless all three of these groups had similar blood ketone levels during the following fasting periods. The same level of ketonemia found in these three groups was present in the animals of Group II-4 after ingesting a greater amount of protein and only traces of fat. Likewise, the animals after a low protein intake and 63 and 30 per cent of their calories as fat (Groups II-1 and II-2) developed similar blood ketone levels. These results are not in accord with the finding of Roberts and Samuels (6) that the degree of fasting ketosis is influenced

TABLE III

Relation of Preceding Protein and Fat Intakes to Fasting Ketonemia

White male rats, Groups II and III, weighing 200 and 150 gm., respectively, were used. The protein and fat intakes per 100 gm. of body weight per day as tabulated are based on the averages for the experimental period of 21 to 23 days. Fasting periods began when the food was removed.

Group and diet No.	No. of animals	Intake per 100 gm.body weight per day		Ketonemia after fasting	
		Protein	Fat	24 hrs.	48 hrs.
		gm.	*gm.*	*mg. per cent*	*mg. per cent*
II-1	8	0.24 ± 0.01	1.91 ± 0.06	20 ± 3	44 ± 3
II-2	8	0.30 ± 0.01	0.89 ± 0.03	22 ± 2	38 ± 2
III-3	9	0.34 ± 0.01	1.36 ± 0.05	11 ± 2	27 ± 2
III-1	9	0.34 ± 0.02	1.38 ± 0.06	10 ± 1	25 ± 2
II-5	8	0.91 ± 0.02	1.82 ± 0.04	7 ± 0	13 ± 2
II-3	8	1.13 ± 0.03	1.38 ± 0.03	9 ± 1	15 ± 1
III-6	10	1.17 ± 0.04	2.45 ± 0.02	7 ± 1	13 ± 2
III-2	8	1.16 ± 0.04	1.11 ± 0.04	10 ± 1	18 ± 2
II-4	8	1.51 ± 0.02	0.10 ± 0.00	10 ± 1	14 ± 2

by the fat content of the preceding diet when the protein intake is the same. In confirmation of earlier work (3), the increase or decrease in liver fat associated with the ingestion of sizable supplements of cystine or choline as in Diets 5 and 2 (Table II) did not affect the fasting blood ketone levels in this study.

Effect of Protein Intake in Preceding Diet—The protein intake appears to be the chief factor governing the following fasting ketosis, as indicated by the results obtained after feeding protein at levels of 5, 20, and 30 per cent of the diet as shown in Table II. Only the animals previously on the 5 per cent protein diets (Nos. 1 and 2) showed a significantly greater fasting ketonemia. The fasting levels after the higher protein diets (Nos. 3 to 6) were similar to those of the fasting controls. The results given in Table III show a definite relation of protein intake to the following fasting

ketone levels.   All levels were similar after the protein intake reached about 1 gm. per 100 gm. of body weight per day.   Group III animals, from a different commercial source, seemed to be somewhat more resistant to developing a marked ketonemia after a low protein intake than did those of Group II.   There were significant increases in ketonemia in all these animals during the second 24 hours on the fast (Table II).   However, the animals of both groups previously on the higher protein and either the low or high fat diets did not reach the same blood ketone levels during the second 24 hours of the fast as did those during the first 24 hours after the lowest protein diet.

MacKay *et al.* (3) have related the protein intake preceding the fast to the degree of fasting ketosis on the basis of the antiketogenic action of the "stored" protein.   The higher nitrogen excretion on fasting after the higher protein intakes was used as a basis for this conclusion.   The better maintenance of the blood sugar as well as the liver glycogen level during fasting in those rats was cited as excellent support for the view.   The increase in the nitrogen excreted amounted to approximately 6 mg. per sq. dm. per day during the 2 day fast.   The antiketogenic material available from the metabolism of protein containing 6 mg. of nitrogen would be equivalent to about 22 mg. of glucose, a very small amount to have such a marked effect upon the metabolism of the fasting animal.

We have shown previously (1) that a rapid drop in the liver glycogen occurs during the early fasting period in animals previously on a low protein diet and suggested an increased rate of carbohydrate utilization in these animals.   Further support for this suggestion was obtained in a recent study (9) in which animals on 5 and 20 per cent protein diets (Nos. 21 and 24) stored 8 and 20 per cent of the caloric intake respectively after similar caloric intakes per unit of body surface.   A significantly greater number of calories were utilized by the animals on the low protein diet. The increase in the degree of ketonemia after the low protein diets might be explained by this increased utilization associated with less gluconeogenesis during fasting.   A more rapid loss of the stores of the more readily available antiketogenic material in these animals would account for an earlier increase in the level of the blood ketone bodies.   Additional evidence is being accumulated which supports this suggestion.

SUMMARY

Various dietary levels of fat, ranging from traces to 63 per cent of the preceding diet, or changes in the fat from 38 to 83 per cent of the caloric intake, with protein intakes the same, produced no significant differences in the blood ketone levels during the following fasting periods.

Low protein intakes were associated with a following fasting ketosis

significantly higher than that after the ingestion of 1 gm. or more of protein per day for each 100 gm. of body weight. The preceding protein intake appears to be the major dietary factor affecting the degree of the following fasting ketonemia.

The effect of the preceding intake upon the fasting ketosis can be explained by an increased utilization of carbohydrate in the animals on a low protein diet as well as by a lack of antiketogenic material during fasting due to the absence of sufficient protein stores.

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PREPARATION OF STREPTOMYCIN

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The announcement of the discovery of a new antibiotic, streptomycin, came early in 1944 (1). This substance was the product of a soil organism, *Actinomyces griseus*. In the short space of time since, a number of trials have demonstrated the value of streptomycin in the treatment of a variety of experimental infections in animals (2–5), and in the clinical treatment of human disease as well (6).

Two publications have appeared, each giving a description for the preparation of a crystalline derivative of streptomycin from highly purified material (7, 8). Schatz *et al.* (1) describe the means of obtaining crude preparations. But no detailed description has appeared of a procedure for obtaining the high purity preparations used in preparing crystalline derivatives and conducting chemotherapeutic trials.[1] The purpose of this paper is to describe a relatively simple procedure for obtaining streptomycin preparations containing 350 to 450 S units per mg. (9).

EXPERIMENTAL

The medium used by Schatz *et al.* (1), a beef extract-peptone broth, added to the difficulties of purifying the antibiotic, chiefly because of materials in the beef extract which caused the preparations from such media to be colored and very hygroscopic. It was found possible to substitute yeast extract for the peptone-beef extract medium.

Data in Table I illustrate experiments comparing four media. These data were obtained by averaging the results of two experiments in which five flasks of each medium were sampled. Figures in Table I represent averages of ten flasks. Of these four media the yeast extract medium, fortified with minerals, gives the most satisfactory production. It is also the best from the standpoint of chemical recovery and consequently was used in our chemical recovery studies. The formula comprises 1 per cent glucose, 1 per cent Bacto-yeast extract, 0.5 per cent NaCl, 0.001 per cent $FeSO_4 \cdot 7H_2O$, 0.025 per cent $MgSO_4 \cdot 7H_2O$; final pH 6.8.

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[1] Since submitting this manuscript, the authors have noted the appearance of a publication concerning purification of streptomycin (Carter, H. E., Clark, R. K., Jr., Dickman, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, **160**, 337 (1945)).

Production—The medium was dispensed into 1 liter (225 ml.) or 2 liter (350 ml.) Erlenmeyer flasks and sterilized. The inoculum was prepared by producing large numbers of spores on agar surfaces in flat sided, large mouthed, 8 ounce bottles. The composition of the sporulating medium was as follows: 1 per cent glucose, 0.05 per cent asparagine, 0.05 per cent K_2HPO_4, 1.8 per cent agar.

A complete mat on the surface of the liquid medium is essential for good production. It was found necessary to use large inocula to accomplish this. After six days incubation at approximately 26° the growth was removed from the agar surfaces by adding sterile, distilled water and

TABLE I

Rates of Production of Streptomycin on Different Media

Age of culture	Streptomycin titer, S units per ml.			
	Medium I	Medium II	Medium III	Medium IV
days				
5	5	21	38	15–20
6	10–15	28	43	25
7	12–18	48	56	35
8	18–22	53	56	38
9	54	76	52	66
10	45	115	74	83
11	43	122	60	76

Medium I, 0.5 per cent peptone, 0.5 per cent beef extract, 0.5 per cent sodium chloride, 1 per cent glucose. Medium II, 1 per cent yeast extract, 0.5 per cent sodium chloride, 1 per cent glucose, 0.001 per cent ferrous sulfate (heptahydrate), 0.025 per cent magnesium sulfate (heptahydrate). Medium III, 0.5 per cent peptone, 0.5 per cent yeast extract, 0.5 per cent sodium chloride, 1 per cent glucose, 0.001 per cent ferrous sulfate (heptahydrate), 0.025 per cent magnesium sulfate (heptahydrate). Medium IV, 1 per cent yeast extract, 0.5 per cent sodium chloride, 1 per cent glucose.

rubbing with a sterile rubber policeman on a glass rod to suspend the spores. The spore suspension was added with a pipette to the surface of each flask. The spores from approximately 2 sq. in. of agar surface were used for each (liter Erlenmeyer) flask. The cultures were incubated at 24–28°. Mats were complete in approximately 48 hours. Flasks were sampled starting at 5 days, when significant activity begins to appear, and samples were assayed. Table II illustrates typical production rates, each figure representing the average of activities shown by five flasks of the same batch. Production begins to level off at approximately 9 days. When maximum activity was reached, the medium was drawn off with suction by inserting a sterile capillary pipette through the mats. Fresh medium, approximately equal in volume, was run in under the mats and incubation continued. As

will be seen in Table II, higher activities were reached in the reflooded flasks after 5 to 7 days than were reached by 10 days in the original medium. This reflooding can be repeated, but is limited by the loss of an occasional flask to contamination and lowered production due to slight but unavoidable damage of the mats each time. It is not generally practical to carry this beyond two refloodings.

In the yeast extract medium the organism produces a red intracellular pigment and a dark brown extracellular pigment which do not appear or are produced in much smaller amounts in the peptone-beef extract medium. These pigments do not interfere with chemical purification, however.

The original medium in the flasks reaches 100 to 200 S units (9) per ml. That obtained by reflooding the cultures reaches 250 to 400 S units per ml.

Chemical Recovery—It is indicated (7) that charcoal adsorption of the antibiotic from the broth and elution with mineral acid are the first steps

TABLE II

Rate of Streptomycin Production in Surface Culture

Original culture medium		Reflooded cultures	
Age of culture	Streptomycin titer	Age from time of reflooding	Streptomycin titer
days	*S units per ml.*	*days*	*S units per ml.*
4½	50	3	140
7	70	4	190
8	100	5	380
9	160	6	314
10	170	7	250

used in chemical recovery by other laboratories. Charcoal is a relatively non-specific adsorbent and a great deal of other material becomes adsorbed and eluted with the streptomycin. An attempt to find a more specific agent among the zeolites failed. Certain of these are capable of adsorbing streptomycin, but the amount of foreign material in the eluates is as high as with charcoal. The other approach was then taken, that of making the charcoal procedure more specific.

The following procedure has been evolved. The broth is filtered free of suspended fragments from the mats. To the clear broth is added 1 per cent of norit A decolorizing charcoal. The broth at this point is approximately pH 8.0. It is stirred mechanically for 20 minutes and the charcoal filtered off on a Büchner funnel with suction. The charcoal is washed with 3 to 4 times its weight of distilled water. This does not result in any loss of streptomycin. The charcoal cake is suspended, with stirring, in methanol to which 0.5 ml. of concentrated hydrochloric acid has been added

per 100 ml.   The volume of acid methanol used is approximately one-tenth that of the original broth.   After 15 minutes the charcoal is filtered off and the methanol neutralized to pH 6.0 with sodium bicarbonate.   (The pH determinations carried out on methanol solutions are actually made on 1:5 dilutions in distilled water.)   The methanol solution is concentrated at 25° and 25 mm. of mercury pressure until the volume has been reduced to 10 per cent of the original.   This solution is treated with 7 volumes of acetone, centrifuged, and the supernatant poured off.   The activity is in the precipitate.   If dried, this material contains 30 to 80 S units of streptomycin per mg.   It is dissolved in distilled water to make a 0.6 to 0.8 per cent solution and adjusted to pH 4.0 with hydrochloric acid.   Then 3 per cent of charcoal is added.   The suspension is stirred for 15 minutes and filtered.   The pH should be checked after mixing in the charcoal, since some charcoal samples contain salts which raise the pH.   The charcoal contains all the colored materials and approximately one-third of the streptomycin.   The clear, colorless filtrate is now adjusted to pH 6.0 by addition of 1 M sodium bicarbonate and mixed, by mechanical stirring, for 15 minutes with 5 per cent of charcoal.   This charcoal is filtered off and washed with 3 to 4 times its weight of distilled water.   The two charcoal cakes are separately suspended in cold methanol, and acid methanol (0.5 ml. of concentrated hydrochloric acid per 100 ml.) added until the pH reaches 3.8.   The volumes are made up with methanol so that the suspension is 20 per cent with respect to charcoal.   Stirring is continued for 15 minutes; then the charcoal is filtered off and washed with a small amount of methanol.   The eluates, with washings added, are neutralized to pH 6.0 with 1 M sodium bicarbonate, evaporated *in vacuo* at 25° to one-quarter the original volume, or less, and precipitated by addition of 7 volumes of acetone.   The suspensions are centrifuged and the supernatants discarded. The precipitates are dried *in vacuo* over concentrated sulfuric acid, and powdered.   That obtained by adsorption at pH 6.0 and elution is white, almost non-hygroscopic, dissolves readily in distilled water, and contains approximately 70 per cent of the activity recovered.   The potency varies from 350 to 450 S units per mg.[2]   The material obtained from the adsorption at pH 4.0 is light brown and is slightly hygroscopic.   It contains streptomycin, 60 to 85 S units per mg., and resembles the crude product from the broth after the first acetone precipitation.   The two crops contain 30 to 35 per cent of the streptomycin in the original broth culture.   Customarily, the recovery crop obtained at pH 4.0 is mixed with those from other batches and reprocessed in the same manner as described (process of

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[2] Use of a Sharples supercentrifuge to remove the norit A from the broth more quickly and additional washing of the charcoal cakes have resulted in increasing the potency of the preparations to as high as 700 S units per mg.

adsorption with pH change repeated). The recovery is approximately 90 per cent with again 70 per cent of the recovered activity in the 350 to 450 unit per mg. fraction.

Example—10.5 liters of medium were drawn off from the mats after **8** days incubation. Assay of this showed 81.5 units per ml., a **total** of 855,000 units. After processing this as described, we obtained 475 mg. of material assaying 400 units per mg. and 1.01 gm. of material assaying 83.5 units per mg.

$$\text{Yield of high potency streptomycin} = 190{,}000 = 22.2\%$$
$$\text{`` `` low `` ``} = 91{,}000 = 10.7\%$$
$$\text{Total recovery} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots = 32.9\%$$

Reprocessing the 83.5 unit material yielded 170 mg. of 348 unit per mg. material and 317 mg. of 72.0 unit per mg. material. Recovery on the reprocessing equals 90 per cent. The yield of high potency material has now increased to 29.1 per cent and a 2.7 per cent recovery of more impure material remains.

Assay—A sample from one of our preparations was submitted to Dr. S. A. Waksman for assay in his laboratory and this salt used as a standard to carry out assays of all broths and other preparations. Our assays have been further checked against a sample of crystalline streptomycin hydrochloride supplied by Merck and Company, Inc.

A plate-cup assay of the United States Food and Drug Administration type with *Staphylococcus aureus* was used. Three dilutions were poured, 25, 50, and 100 S units per ml. The solutions for assay were made up in a 0.05 M sodium phosphate buffer at pH 7.0. The medium used was that recommended by the United States Food and Drug Administration for penicillin assay. Twelve plates were used for each assay, four cups on a plate. Thus there are eight cups at each dilution for both standard and unknown.

The zones are smaller than those obtained for assays of penicillin or *Bacillus subtilis* streptomycin assays, but are very clear and have sharp boundaries. The three zone sizes plotted on semilogarithmic graph paper give a straight line.

Example—A factorial analysis was carried out on several assays according to the method of Bliss and Marks (10). This was found satisfactory from the standpoints of possessing parallelism of standard and unknown curves, no opposed curvatures, and adherence to straight line relations between log-dose and response. In a typical analysis the unknown was calculated to 118 per cent of the standard with limits of 114 to 122 per cent.

Toxicity—A carotid blood pressure recording was made on a 5.5 kilo dog under sodium barbital anesthesia. Solutions of two streptomycin

FIG. 1. Carotid blood pressure recording of a 5.5 kilo dog under barbital anesthesia, with injections of streptomycin in the femoral in at the points indicated.

preparations were made up to contain 20 mg. per ml. Preparation 1 was 348 units per mg.; Preparation 2 was 400 units per mg. Injections were made into the femoral vein, first 1 ml. of Preparation 1 and after an interval of 5 minutes, another 4.0 ml. of Preparation 1. After a 10 minute interval 1 ml. of Preparation 2 was injected, then 4.0 ml. The blood pressure recordings are presented in Fig. 1. It is seen that both the salts were without significant effect on the blood pressure, though the sum total of injections in a 20 minute period corresponds to far larger amounts than are customarily given to human patients on a body weight basis (6). The dog recovered well after the slit carotid artery was sewed up. Early streptomycin preparations exhibited a "histamine effect" (11). This experiment demonstrates that the histamine-like factor is not present in these preparations.

Toxicity studies were made on some of our streptomycin preparations by intravenous injection in mice. The preparations were made up at various concentrations in distilled water and injected into the tail veins of white mice weighing 20 gm. One preparation, 400 S units per mg., pH 7.1, was tolerated at 5.0 mg. (2000 S units), but killed the mice, without exception, at 7.5 mg. Another preparation, 500 S units per mg., pH 7.0, killed one mouse of five at 5.0 mg. (2500 S units) and killed all mice injected with 6.0 mg. (3000 S units). No difference was observed when smaller volumes were used (0.2 ml. injections instead of 0.5 ml.).[3]

When the intravenous injections are made, there is an immediate and violent reaction with respiratory difficulty. The mice either die within 5 minutes or survive indefinitely. The tolerance is approximately 5 times higher if the injection is made intraperitoneally. It is evident that a more rigorous study of the toxicity testing of streptomycin preparations is needed.

Denkelwater et al. (12) have demonstrated that cysteine inactivates streptomycin, but is without effect on streptothricin. Incubation of 400 S units of one of our streptomycin preparations with 2 mg. of cysteine for 1 hour at 37°, pH 7.0, completely inactivated the preparation.

DISCUSSION

The medium used in this work for streptomycin production has the disadvantage that it costs approximately 50 per cent more than does the peptone-beef extract medium. This is far outweighed by the advantages of higher, more rapid production, together with greater ease in purification of the product. The peptone-beef extract medium gives preparations of almost equal potency after additional manipulations, but they still contain

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[3] Preliminary testing, since crystalline streptomycin hydrochloride has become available to us, indicates that the toxicity of our preparations is all due to the streptomycin, and not to any impurities.

colored materials. Their toxicity has not been studied. Batches of streptomycin broth produced in Media II, III, and IV, listed in Table I, of equal volume and approximately equal potency, were processed under identical conditions. The yields were approximately equal. The potencies of the products were as follows: Medium II 368 S units per mg., Medium III 248 S units per mg., Medium IV 264 S units per mg.

Broths of potencies varying from 60 to 380 S units per ml. have been processed in the manner described. There appears to be no relation between potency of the broth and potency of the final product. Broths at both extremes, treated in an identical manner, both yielded products which were 400 S units per mg. Final products of lower potency are encountered if the charcoal is left in contact with the broth too long, when it adsorbs additional foreign matter.

During hot weather, when incubator space could not be held below 30°, it was found that this variance from the optimum conditions for the organism caused a marked fall in production of streptomycin.

The chief impurity in our preparations is sodium chloride. This enters as a result of the back neutralization necessary after elution with acid methanol. This impurity is no disadvantage to the therapeutic use of the preparations.

SUMMARY

A procedure for production of streptomycin in surface culture is described. A new method for purification of the antibiotic from these cultures is outlined. The products have a potency of 350 to 450 S units per mg.

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LETTERS TO THE EDITORS

ON THE FUNCTION OF PYRIDOXINE IN LACTIC ACID BACTERIA

Sirs:

During the course of our studies on the use of microorganisms for the determination of amino acids, we have found that the amino acid requirements of several lactic acid bacteria are modified not only by pyridoxine and pyridoxine derivatives, as reported by Stokes and Gunness,[1] but also by the presence or absence of CO_2.

It was observed that the growth response of the organisms grown in a medium containing pyridoxine but deficient in any one of several amino acids was rapid when the cultures were grown in tubes of small diameter, while only scant growth occurred in Erlenmeyer flasks in which the relative surface area was much larger. This difference in growth response was found to be due to a difference in the amount of metabolic CO_2 retained in the culture medium.

The results of an experiment which shows the relationship of both pyridoxine and CO_2 to the amino acid requirements of *Streptococcus faecalis* R and *Lactobacillus arabinosus* are given in the table.

The cultures were grown in 50 ml. Erlenmeyer flasks. In the tests with carbon dioxide the cotton stoppers were replaced with sterile rubber stoppers after seeding. Then by the use of sterile hypodermic needles and cotton filters the air in the flasks was displaced by flushing with 2 liters of a gas mixture containing 6 per cent CO_2 and 94 per cent air. The needles were removed from the stoppers before incubating the cultures. The medium was similar to that used for amino acid assays.[2] Succinic acid was used as a buffer in the *Streptococcus faecalis* R medium. When pyridoxine was used, it was autoclaved with the medium.

Since CO_2 is not required for the growth of the organisms in a medium containing all of the amino acids, it is probable that CO_2 is required specifically for amino acid synthesis. Likewise, since pyridoxine or pyridoxine derivatives are not required for rapid growth in a medium containing all the amino acids, but are required in a medium with certain amino acids omitted, it is probable that some form of pyridoxine is involved in an

[1] Stokes, J. L., and Gunness, M., *Science*, **101**, 43 (1945).
[2] Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).

DEHYDROPEPTIDASE ACTIVITY IN TISSUES

Sirs:

Peptides of α-aminoacrylic acid (dehydroalanine) may be enzymatically hydrolyzed to products which include pyruvic acid and ammonia.[1] An active dehydropeptidase for chloroacetyldehydroalanine is present in ex-

*Hydrolysis of Dehydropeptides in Tissue Extracts**

Tissue	Ammonia N liberated from							
	Chloroacetyldehydroalanine				Glycyldehydroalanine			
	Rat	Mouse	Rabbit	Guinea pig	Rat	Mouse	Rabbit	Guinea pig
Liver	20†	15	12	12	20†	18	13	15
Fetal liver	5		3		20		12	
Primary hepatoma	10				20			
Transplanted hepatoma	1	0			20	18		
Spleen	0	0	0	0	19	17	12	16
Kidney	20	23	14	16	22	21	14	19
Brain	0	0	0	0	18	17	15	20
Muscle	0	0	0	0	20†	16	12	14
Pancreas	10	4	3	10	17	20	15	15
Intestinal mucosa		2				20		
" adenocarcinoma		0				19		
Blood serum‡	0		0	0	11		14	14

* The data are given in terms of moles × 10⁻⁶ of ammonia N liberated from 25 × 10⁻⁶ mole of substrate in 1 cc. after 2 hours incubation at 38° with 1 cc. of phosphate buffer at pH 6.9 and 1 cc. of tissue extract equivalent to 166 mg. of tissue. The results are corrected for appropriate blanks. Nearly identical results were obtained under anaerobic conditions.

† Pyruvate was present to the extent of 16 to 18 moles × 10⁻⁶. Maximum hydrolysis of substrates corresponding to 20 to 22 moles × 10⁻⁶ of ammonia N was reached in 2 hours; 50 per cent hydrolysis was reached after about a half hour of incubation. Neither glycine nor glycylglycine yields ammonia or pyruvate under present conditions.

‡ 1 cc. of serum was used for the digests. Corresponding values for human serum are 0 with chloroacetyldehydroalanine and 4 with glycyldehydroalanine.

tracts of liver, kidney, and pancreas of rats, mice, rabbits, and guinea pigs;[2] under the same conditions this substrate is not appreciably attacked in the serum or in extracts of spleen, brain, muscle, or tumors of these species.

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We have recently observed that when glycyldehydroalanine is used as the substrate every tissue studied, whether normal or neoplastic, actively hydrolyzes it (see the table). It would appear that there are at least two dehydropeptidases, one with a very wide distribution among tissues which may be designated dehydropeptidase I and the substrate for which is glycyldehydroalanine, and the other with a relatively limited distribution among tissues which may be designated dehydropeptidase II, the substrate for which is chloroacetyldehydroalanine. The former enzyme, unlike the latter, apparently requires the presence of a free α-amino group in the acyl residue of the substrate.

The wide-spread occurrence and powerful activity of dehydropeptidase in tissues, as compared with the relatively weak activity intracellularly of the peptidases for saturated chain peptides (aminopeptidase, carboxypeptidase, etc.), lend weight to Bergmann's original view[3] that amino acids can be catabolized when in peptide linkage. The occurrence of dehydropeptidase I in tissues lacking exocystine desulfhydrase activity[2] suggests that the precursors of α,β-unsaturated amino acid residues in susceptible dehydropeptides may be other amino acids besides cystine.

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A SUGGESTED MODIFICATION OF THE MICROBIOLOGICAL ASSAY FOR RIBOFLAVIN

Sirs:

A number of reports[1] have called attention to the fact that natural sources of the vitamin B complex contain substances other than the vitamins that stimulate the growth of the microorganism, *Lactobacillus casei*, used in microbiological assay procedures. In the riboflavin assay procedure the non-riboflavin stimulatory effect becomes significant when the lower levels of added test solution give an apparent riboflavin content appreciably in excess of that shown by the larger additions.

In our experience careful removal of the protein and fat fractions of the test solution has eliminated the non-specific stimulatory effect. The U. S. P. microbiological assay procedure[2] provides for the removal of these fractions, but a more detailed description is desirable for application to food products and particularly cereals. After the sample has been treated with 0.1 N HCl in the autoclave for 30 minutes and cooled to room temperature, as directed in the U. S. P. procedure, the suspension is adjusted to pH 5.5 to 6.0 with NaOH solution and HCl is immediately added until no further precipitation occurs (at the isoelectric point of the protein, about pH 4.5). The suspension is diluted to a measured volume such that it contains more than 0.1 γ of riboflavin per ml. and filtered through paper known not to adsorb riboflavin. Dissolved protein, if present, is detected in the clear filtrate, at a pH both below and above that first used, by adding dropwise first HCl solution then NaOH solution. If further precipitation occurs, the solution must again be adjusted to the point of maximum precipitation and then filtered. To a measured volume of the clear filtrate NaOH solution is added to a pH of 6.6 to 6.8. If cloudiness occurs, the solution is again filtered, and then diluted to a final volume calculated to contain 0.1 γ of riboflavin per ml.

In the table data are presented showing the stimulatory effect that may occur when the protein fraction has not been removed completely from the test solution. A comparison is made of values obtained for the different

[1] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939). Bauernfeind, J. C., Sotier, A. L., and Boruff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 666 (1942). Andrews, J. S., Boyd, H. M., and Terry, D. E., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 271 (1942). Williams, V. R., and Fieger, E. A., *Cereal Chem.*, **21**, 540 (1944); *Ind. and Eng. Chem., Anal. Ed.*, **17**, 127 (1945).

[2] The pharmacopoeia of the United States of America, Twelfth decennial revision, 1st bound supplement, Easton (1943).

levels of test solution (*a*) after precipitation with alkali and (*b*) after precipitation according to the modified procedure.

Material assayed	Method of pptn.*	Riboflavin at different levels of test solution		
		1.0 ml. test solution	1.5 ml. test solution	2.0 ml. test solution
		γ *per ml.*	γ *per ml.*	γ *per ml.*
Vitamin B complex capsules	(*a*)	0.110	0.096	0.094
	(*b*)	0.094	0.092	0.094
Dried brewers' yeast	(*a*)	0.092	0.088	0.083
	(*b*)	0.082	0.082	0.081
Enriched flour	(*a*)	0.099	0.093	0.091
	(*b*)	0.088	0.089	0.088
" bread	(*a*)	0.101	0.095	0.085
	(*b*)	0.086	0.084	0.085

(*a*) precipitation with alkali; (*b*) precipitation according to the modified procedure.

By approaching the isoelectric point from the alkaline side, more nearly complete removal is assured. The fraction that stimulates is readily soluble above but insoluble below the isoelectric point over a fairly wide range of acid concentration. The fat fraction is removed satisfactorily by the process of filtration through paper.

Food and Drug Administration
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H. W. LOY, JR.
O. L. KLINE

REVERSAL BY TRYPTOPHANE OF THE BIOLOGICAL EFFECTS OF 3-ACETYLPYRIDINE

Sirs:

Woolley[1] has shown that the feeding of 3-acetylpyridine (a structural analogue of nicotinic acid) to mice caused the production of a fatal disease with many manifestations similar to those of human pellagra or of canine blacktongue. This action of 3-acetylpyridine could be negated completely by addition of nicotinic acid to the diet. At about the same time, Krehl *et al.*[2] demonstrated that the inclusion of corn in a highly purified basal ration for rats brought about retardation of growth and that this deleterious effect of corn could be overcome either with nicotinic acid or with tryptophane. These authors postulated a selective .action of the corn on the intestinal flora as an explanation of their findings. However, an alternate hypothesis is that the "pellagragenic" action of corn may be related to the occurrence in it of a structural analogue of nicotinic acid which competes with that vitamin just as 3-acetylpyridine does. Since tryptophane as well as nicotinic acid counteracted the effects of corn, it was decided to determine whether tryptophane would also overcome the action of 3-acetylpyridine. The experiments described below will show that tryptophane did nullify the toxic effects of 3-acetylpyridine.

Responses of Mice to Added Tryptophane and 3-Acetylpyridine

dl-Tryptophane added	3-Acetylpyridine	No. of animals	No. of deaths	Average change in weight
per cent	*mg. per day*			*gm. per wk.*
0	0	12	0	+4.0
0	4	12	11	
2.0	4	6	0	+5.2
1.0	4	11	1	+6.1
0.3	4	11	1	+6.1
0.1	4	6	1	+4.6

Weanling mice were caged individually on screen floors and fed a diet composed of sucrose 75 gm., casein 18 gm., salts[3] 5 gm., heated starch (amigel)[4] 20 gm., fortified corn oil[5] 1 gm., thiamine 0.2 mg., riboflavin 0.5

[1] Woolley, D. W., *J. Biol. Chem.*, **157**, 455 (1945).

[2] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101,** 489 (1945).

[3] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109,** 657 (1935).

[4] Supplied by the Corn Products Refining Company.

[5] Woolley, D. W., *J. Biol. Chem.*, **143,** 679 (1942).

mg., pyridoxine 0.2 mg., calcium pantothenate 2 mg., choline chloride 10 mg., and inositol 100 mg. To this basal ration various amounts of tryptophane were added. Beginning on the 4th day each animal was given orally 4 mg. of 3-acetylpyridine per day. The experiment was continued for 10 days, and the animals were observed for signs of disease and were weighed twice weekly. In one experiment, to verify the previous finding that this vitamin would protect the animals,[1] a group of six mice was fed the basal ration plus 0.5 per cent of nicotinamide.

The data in the table show that tryptophane nullified the toxicity of 3-acetylpyridine. It also prevented the pellagra-like manifestations called forth by the ketone. As little as 0.1 per cent of the amino acid was sufficient to protect the animals. Therefore, tryptophane was as active in this respect as was nicotinic acid.[1] In view of these results and those of Krehl *et al.*[2] it might be of interest for a study of the etiology of pellagra to examine corn for the presence of a structural analogue of nicotinic acid.

The Rockefeller Institute for   D. W. WOOLLEY[6]
   Medical Research
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THE COMBINATION OF FATTY ACIDS AND RELATED COMPOUNDS WITH SERUM ALBUMIN

I. STABILIZATION AGAINST HEAT DENATURATION

By PAUL D. BOYER, FUNSTON G. LUM, GERALD A. BALLOU, J. MURRAY LUCK, AND RANDALL G. RICE

(From the Biochemical Laboratory, Department of Chemistry, Stanford University, California)

In a recent publication (1) data were presented which showed that lower fatty acid salts markedly retard the heat coagulation of concentrated serum albumin solutions, and a simple "cloud point" method for evaluation of the relative stabilization produced by different substances was described. These experiments have been extended to include studies of the thermal stability, as measured by nephelometric, viscometric, and cloud point methods, at different concentrations of stabilizers and albumin, and in the presence of a number of related compounds. The results and conclusions from these later studies are reported herein.

Methods

The serum albumin solutions were prepared as described previously (1). Amorphous and crystalline human and bovine albumin were used. The crystalline human and bovine albumin preparations were homogeneous as determined by electrophoretic analysis; the amorphous bovine albumin contained about 2 per cent of α-globulin, and the amorphous human preparations contained from 1 to 2 per cent of globulin. The behavior of these various serum albumin preparations in the presence of stabilizing agents was quite similar.

The pH of the albumin solutions, with or without the addition of substances tested for stabilizing activity, was between 6.8 and 7.5 unless otherwise noted. Cloud point and nephelometric measurements were made as previously described (1, 2). Viscosity determinations were made with Ostwald viscosity pipettes, with a constant temperature bath regulated to ±0.01°. The water times at 30° of the pipettes used with 2 gm. per cent solutions of albumin were from 60 to 70 seconds, and the water times of the pipettes used with 25 gm. per cent solutions of albumin were from 7 to 10 seconds. The pipettes were thoroughly cleaned with dichromate cleaning solution between determinations. The test solutions were heated in stoppered viscometers, which were opened when a determination was made. The time of heating for a particular viscosity determination was

taken as the time during which the sample had been in the water bath before the viscosity measurement was begun plus one-half of the drainage time for the pipette. Viscosity measurements on a solution were discontinued if any particulate material, observable in a strong cross-beam of light, appeared.

. Most of the experiments reported herein were conducted with solutions containing 25 gm. per cent of albumin, because of the relation of the studies to the practical problem of thermal stabilization of albumin solutions as prepared for the armed forces. Although theoretical studies with concentrated protein solutions are necessarily limited, relative data on the thermal stability of the albumin are readily obtainable with such solutions.

RESULTS AND DISCUSSION

Cloud Point Studies with Various Compounds—The effects of increasing concentration of the sodium salts of straight chain fatty acids on the cloud point are shown in Fig. 1. The data given in Fig. 1 as well as in Figs. 2 and 3 were obtained with 25 gm. per cent solutions of a crystalline human serum albumin preparation. The cloud point temperature was taken as the temperature at which a coagulum formed in 30 ± 2 seconds when the solutions were heated in capillaries at constant temperature (1). The increase in stabilization with increase in chain length of the fatty acid salt, as noted previously with relatively high concentrations of the fatty acid salts (1), is clearly evident. Maximum stabilization is attained with salts of fatty acids 7 to 8 carbon atoms in chain length and in a concentration of about 0.15 M. Above this concentration gel formation ensues. Below a concentration of about 0.015 M, stabilization increases with increase of chain length up to the 12 carbon acid but the effect falls off with increase of concentration. This was the longest chain fatty acid studied; the low solubility of the sodium salts of fatty acids of greater chain length did not permit their use. Fig. 1 serves to illustrate the relationship between chain length of the fatty acid salt, its concentration, and the degree of stabilization attained. The diminishing efficacy of the higher fatty acid salts (above C_8) is probably due to the denaturing effects of substances with detergent properties (3–5). Dodecyl sulfate, which has been observed to cause denaturation of serum albumin (5, 6), has a stabilizing effect in low concentrations similar to that produced by laurate. As the concentration of the fatty acid salts with 7 or more carbon atoms is increased, a point is reached at which a cloud no longer forms, but instead a clear gel results at the elevated temperature. The concentration at which this phenomenon may be noted decreases with increase in the length of the fatty acid chain.

Measurements of the cloud point temperature of albumin-fatty acid salt mixtures at different time intervals after mixing showed that the cloud

point had already attained a constant value when the measurements were made as soon as possible after mixing. Thus, in so far as the cloud point of a solution serves as a measure of the amount of combination of a particular fatty acid with the albumin, a conclusion which seems reasonably justified on the basis of experiments described later, the combination between the fatty acid and the serum albumin proceeds at a very rapid rate. Ultrafiltration measurements also show that the amount of combination reaches a constant value within the time necessary for the experiment.[1]
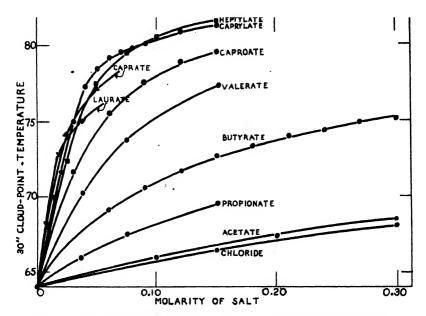
Fig. 1. The stabilization of serum albumin to heat by fatty acid salts

Experiments with alkyl sulfonates, given in Fig. 2, showed that these substances likewise have a pronounced protective effect against heat coagulation, and that this effect increases with the size of the alkyl groups. The curves for butyrate and caprylate are included for comparison. The effect of the sulfonates is not quite as great as that of the fatty acid salts on the basis of molecular size; *e.g.*, caproyl sulfonate is a slightly larger molecule than heptylate, but is slightly less effective. Although these slight differences were noted, the relative effects of the sulfonates and carboxy acids with alkyl groups of the same chain length do not differ much, in-

[1] Boyer, P. D., Ballou, G. A., and Luck, J. M., unpublished.

dicating that the strength of the acid group does not markedly affect the amount of stabilization, since the sulfonic acids are highly dissociated, while the carboxy acids have pK values of about 4.9 to 5.0 (7, 8). That the strength of the acidic group is not a primary factor is also evident from the comparative effects of the fatty acids, all of which have about the same dissociation constant.
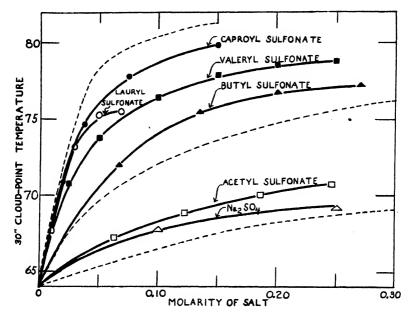
FIG. 2. The stabilization of serum albumin to heat by alkyl sulfonates. The dotted curves represent butyrate and caprylate.

The effects of various aromatic acids on the cloud point are shown in Fig. 3. The addition of a phenyl group to a fatty acid anion markedly increases the stabilizing effect of a fatty acid, although the phenyl group has less effect than an alkyl group of the same number of carbon atoms.

The addition of a hydroxyl group to a compound results in a definite decrease in the stabilizing effect. Comparisons between propionate and lactate, butyrate and β-hydroxy butyrate, and phenyl acetate and mandelate demonstrated that the compounds with hydroxyl groups are less effective. Sodium gluconate also had but slight effect.

The presence of additional carboxyl groups likewise decreases the protective action of a compound. Sodium succinate and fumarate are far less effective than propionate and butyrate, and are even less effective than chloride. Phthalate is also considerably less effective than benzoate.

Several other compounds of interest have been tested and found effective as stabilizing substances. Trichloroacetate and tungstate, which in the acid state are commonly used as protein precipitants, increase the thermal stability. Trichloroacetate (0.0375 to 0.15 M) was more effective than tungstate (0.0375 to 0.15 M); the effect of the former is similar in degree to that of caproate; tungstate is about as effective as acetate. Monochloro-
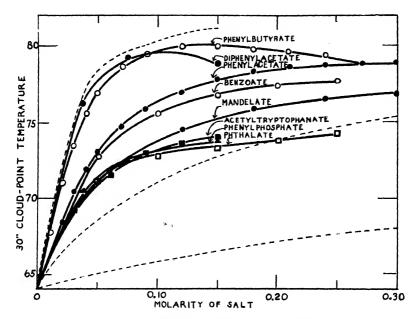
FIG. 3 The stabilization of serum albumin to heat by salts of various aromatic acids.

acetate is much less effective than trichloroacetate, although more effective than acetate. Sodium salts of cholic, desoxycholic, and dehydrocholic acids in concentrations up to 0.025 M have a protective action of about the same magnitude as butyrate. Of these salts the desoxycholate is the most effective. Formaldehyde also produces an increase in the cloud point temperature, although its mode of combination with the albumin is un-doubtedly different from that of the fatty acids. Butyraldehyde, although possessing a non-polar side chain, produces much less effect than the more reactive formaldehyde. The increased stability of serum albumin in the presence of formaldehyde has been noted previously (9). The observation of Spiegel-Adolf that cephalin will prevent the heat coagulation of serum albumin (10) is also of interest.

The importance of negatively charged groups in the stabilizing sub-

stances was shown by experiments in which non-charged compounds with non-polar groups were used.   The solubility of such compounds is small, but those studied had sufficient solubility so that solutions were readily obtained with concentrations comparable to the effective concentrations of anions.   Ethyl butyrate, butyramide, caproamide, monocaproin, monocaprylin, monocaprin, and triacetin all have only small stabilizing effects with crystalline human serum albumin solutions; none of the compounds has an effect as great as that of butyrate; most of them are roughly as effective as chloride.   With amorphous albumin preparations, a more pronounced stabilization was sometimes noted with monocaproin, but the results were erratic and always less than the consistent stabilization produced by the fatty acid salts.

In contrast to the effect of anions, cations with non-polar groups increase the susceptibility of serum albumin to heat denaturation.   At pH 7 to 8, additions of dodecylamine and hexylamine hydrochloride to 25 gm. per cent solutions of albumin decreased the cloud point temperature in proportion to the concentration of the amine.   0.15 M solutions produced decreases of 10° and 4°, respectively.

Cloud point experiments on the acid side of the isoelectric point of albumin showed that the opposite effect of cations and anions is not due to the respective decrease and increase in the negative charge which their combination with the albumin would produce.   A 25 gm. per cent solution of crystalline bovine albumin which contained 0.05 M NaCl and had been brought to pH 4.3 by addition of acetic acid had 30 second cloud point temperatures of 63.5°, 62.0°, 60.0°, and 59.0° in the presence of 0.025 M caprylate,[2] 0.05 M caproyl sulfonate, no addition, and 0.05 M hexylamine respectively.   Thus, stabilization by anions is evident on both the alkaline and acid side of the isoelectric point.   .

A variety of other compounds has been tested.   All of these had slight stabilizing effects in comparison to the pronounced effects of low concentrations of fatty acid salts and related compounds.   The compounds tested were glycine, alanine, glycylalanine, alanylglycine, gelatin, α-globulin, glycerol, glycerophosphate, xylose, glucose, maltose, sucrose, α-methylglucoside, ascorbic acid, sodium phosphate, and sodium sulfate.

Requisite Structure for Stabilizing Properties—The above experiments permit definition of the structure of a group of compounds which will stabilize serum albumin against heat denaturation and coagulation.   The basic, requisite structure is an anion with a non-polar group.   Of forty different compounds of this type, including derivatives of carboxylic,

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[2] In aqueous solution at this pH, caprylic acid exists principally in the undissociated, water-insoluble form.   However, in the concentrated albumin solutions used no separation of free caprylic acid was noted.

sulfonic, sulfuric, and phosphoric acids, all had stabilizing properties in low concentrations. Twenty-three other compounds, including amino acids, peptides, proteins, sugars, alcohols, amides, esters, and amines with non-polar groups, did not possess the marked stabilizing properties of the non-polar anions. The magnitude of the stabilization produced by any type of anion increases in general with the size of the non-polar group until a maximum is reached. The addition of hydroxyl or carboxyl groups to the non-polar groups, with the consequent increase in polarity of the group, reduces the effect of any given anion.
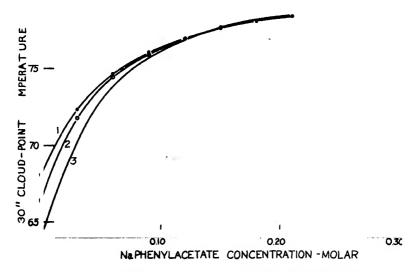
FIG. 4. The effect of variation in the ionic strength on the stabilization produced by phenyl acetate. Ionic strength maintained at 0.30 by additions of NaCl (Curve 1); constant amount of 0.15 M NaCl (Curve 2); phenyl acetate alone (Curve 3).

Variations in Ionic Strength and Albumin Concentration—In the preceding experiments the ionic strength of the solutions increased with increasing concentrations of the sodium salts of the anions. However, the ionic strength is not an important factor in determining the degree of stabilization. In Fig. 4 are shown results of experiments with increasing concentrations of phenyl acetate with the ionic strength maintained constant at 0.30 by NaCl additions (Curve 1); the presence of a constant amount, 0.15 M, of NaCl (Curve 2); and, no additions other than phenyl acetate (Curve 3). These results show that at low concentrations of phenyl acetate, the effects of chloride and phenyl acetate were additive. As the phenyl acetate concentration increased, the effect of chloride dis-

appeared, and with concentrations above about 0.12 M the cloud point temperature was determined only by the concentration of phenyl acetate, and was independent of the amount of sodium chloride.

The cloud point temperatures of albumin solutions decrease with increase in protein concentration (1); hence it was of interest to determine the effect of fatty acid salts at different albumin concentrations. Results obtained with crystalline human albumin solutions, given in Fig. 5, show
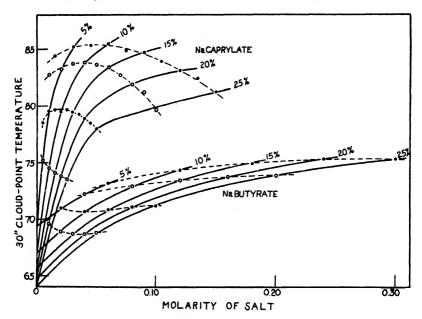
FIG. 5. The effect of variation in the albumin and the fatty acid anion concentration on the cloud point temperature. The solid curves show the cloud point temperatures at the albumin concentrations indicated. The dotted lines show the effect of dilution of a given solution with water, thus maintaining a constant mole ratio of stabilizer to albumin.

that at the concentrations of albumin studied the fatty acid salts have the expected marked stabilizing effect. The curves obtained with a constant ratio of stabilizer to albumin represent the summation of the opposing effects of decreasing both the albumin and fatty acid concentrations. Calculated curves approximating the experimental curves were obtained by algebraic addition of the changes in the cloud point temperature resulting from the reduction of albumin concentration with the salt constant at 0.3 M and the changes produced by reduction of the fatty acid salt concentration with albumin constant at 25 gm. per cent. This interpretation also explains the constant mole ratio curves previously presented (1).

Effect of Caprylate at High pH—The amount of bound caprylate decreases with increase in the pH of an albumin-caprylate mixture.[1] Determinations of the cloud point temperatures of solutions containing 5 gm. per cent of bovine albumin in the presence and absence of caprylate, and with increasing amounts of sodium hydroxide added are shown in Fig. 6. The similarity of the cloud point temperatures of the two solutions at the high pH value is probably due to the decrease in the amount of
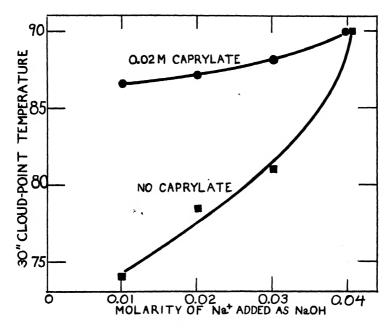
FIG. 6. The effect of increase in pH on the stabilization produced by caprylate. The cloud points were obtained with solutions containing 5 gm. per cent of bovine albumin to which increasing amounts of NaOH had been added. The pH of the solutions was 6.8, 8.1, 10.3, and 10.8 with the addition of 0.01, 0.02, 0.03, and 0.04 M Na$^+$ as NaOH. At higher concentrations of NaOH, the solutions formed clear gels when heated.

bound caprylate, as well as to the effect of the alkali on the coagulation of the albumin.[3]

Limitations of Cloud Point Method—The principal value of the cloud point method has been in comparative studies of the effects of various

[3] This conclusion is based on the assumption that the amount of caprylate bound at cloud point temperatures is the same as that bound at room temperature. This assumption appears justified because the amount of bound caprylate does not change between 0–50° (Boyer, Ballou, and Luck, unpublished data).

substances on the thermal stability of albumin solutions under well defined conditions and as a convenient, rapid method for evaluation of the relative stability of different albumin solutions. Theoretical conclusions which can be made from cloud point data are definitely limited. For example, a variation in crystalline albumin concentration from 20 to 30 gm. per cent changed the cloud point time at constant temperature from 170 to 18 seconds. If the reciprocal of the cloud point time is assumed to be directly proportional to the velocity of the reaction, then an order for the denatura-tion reaction can be calculated. However, such calculations give the anomalous value of a fifth or sixth order reaction. Likewise, the energy of activation for the denaturation-coagulation reaction can be calculated if the velocity constant is assumed to be proportional to the reciprocal of the cloud point time. As shown previously (1), the calculated values exhibit marked variations with the nature of the substance added and with the protein concentration, as well as the expected variations with pH. That such values are open to question is further shown by the variation in the slope of the straight line obtained from a plot of the log of the cloud point time against concentration of added salt. For example, with phenyl acetate irregular variations of slope ranging from -0.55 to -0.90 were noted with seventeen different concentrations of phenyl acetate ranging from 0 to 0.30 M. The inadequacy for kinetic studies of methods based on flocculation of proteins has been pointed out by other investigators (11).

Nephelometric Measurement of Stabilization—The protection from heat denaturation afforded by the addition of low concentrations of various substances may also be measured by nephelometry. The effects of various mole ratios of caprylate, mandelate, or laurate to albumin on the stability of 25 gm. per cent solutions of human serum albumin at 57° are shown in Fig. 7. Caprylate and laurate have about the same effect, and show a detectable stabilization when present in a concentration of only 1 molecule for every 4 albumin molecules. At a 1:1 ratio, caprylate produces definite stabilization, and is about 4 times as effective as mandelate. The remarkable effect of low concentrations was also shown by the cloud point studies. Addition of sufficient caprylate to give a caprylate to albumin ratio of 1:1 increased the cloud point time at constant temperature by about 10-fold.

In previous studies of nephelometric stability at 50° and 57°, caprylate and caproate in concentrations of 0.15 M did not always exhibit the stabilization which was expected on the basis of cloud point results (1). These rather anomalous findings are now known to have resulted from the presence of very small amounts of proteins other than native albumin which were flocculated by the relatively high concentrations of the fatty acid salts. If these impurities were removed by heating the solution for a short time in the presence of low concentrations of the fatty acids,
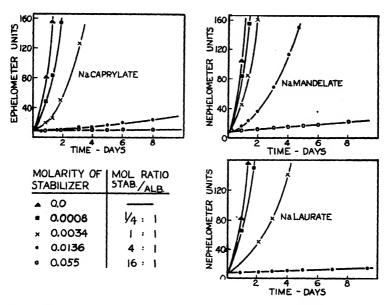
FIG. 7. Effective mole ratios (stabilizer to albumin) for stabilization of serum albumin.
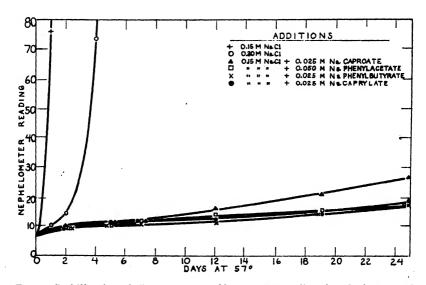
FIG. 8. Stabilization of 25 gm. per cent of human serum albumin solutions at 57°

followed by filtration, the resulting products possessed high nephelometric stability. Typical results of the stabilization of a preparation which did not contain impurities readily flocculated are shown in Fig. 8. Prolonged

studies at 57° revealed that the fatty acid salts increase in protective properties with increase in chain length.   Comparisons of the stabilizing properties at 50° were limited because of the long time period required. Results of a study extending over a period of 20 months are given in Table I. The values represent time for gel formation rather than time to reach a certain nephelometric reading, because some of the samples formed gels without showing high nephelometric readings.   Stabilization by the fatty acid salts at 50° is clearly evident, although the relative life spans of the solutions at 50° are not as widely different as at cloud point temperatures; for example, the 65° cloud point time (1) of serum albumin in 0.15 M NaCl was 63 seconds, while in 0.025 M sodium caproate it would appear to be

TABLE I

Stability of Albumin Solutions at 50°

All samples contained 25 gm. per cent of amorphous human serum albumin and 0.15 M sodium chloride, and were stored in an air oven at 50° until the solution formed a gel.

Salt added	30 sec. cloud point temperature	Mos. at 50° for gel formation	Appearance of gel
	°C.		
None............................	63.1	3	Opaque
0.15 M NaCl.....................	65.4	7	"
0.025 M Na caproate............	70.6	13	Cloudy
0.025 " " phenyl butyrate....	72.6	13	Nearly opaque
0.15 " " butyrate............	71.7	16	" "
0.05 " " phenyl acetate......	72.7	16	Partly cloudy
0.025 " " caprylate...........	73.5	18	Nearly clear
0.15 " " phenyl acetate......	76.9	20	" "
0.15 " " phenyl butyrate ...	81.5	20	" "

even greater than 20,000 seconds.   The corresponding 50° stability values were 7 and 13 months respectively.

Viscometric Measurement of Stabilization—In order to ascertain whether changes which did not produce light scattering took place in the albumin molecule when solutions were heated in the presence of fatty acid salts, viscosity measurements were made on the heated solutions.   Viscometric measurements of the stability of serum albumin in the presence of sodium chloride have been reported by Scatchard *et al.* (2).   Cooper and Neurath (12) and Pedersen (13) have shown that heat treatment of crystalline horse serum albumin solutions results in an aggregation of the albumin and an increase in viscosity.

In our studies, viscosity measurements were made at the temperature of denaturation to eliminate any effects of subsequent cooling of the solu-

tion. The viscosity increase that resulted when solutions containing 25, 20, and 15 gm. per cent of amorphous bovine albumin were heated at 57° is shown in Fig. 9. The relative viscosities of the solutions at zero time were ascertained by measurement at 50°, a temperature at which viscosity increase would occur only after prolonged periods. The pronounce.. effect of protein concentration is clearly evident. The addition of 0.025 M caprylate prevented any viscosity increase during the 24 hour period of measurement. Thus caprylate prevents changes in the albumin molecule which give rise to change in viscosity. These results indicate that caprylate stabilizes albumin in the native state, rather than prevents the aggregation
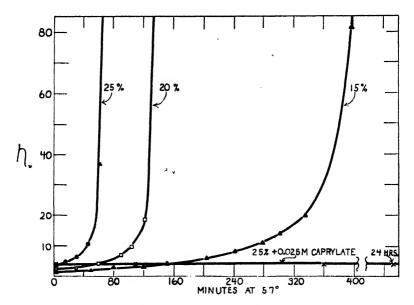
FIG. 9. Viscosity of concentrated serum albumin solutions

of the heated albumin into light-scattering particles. These conclusions were substantiated by experiments at lower protein concentrations. Results of viscosity measurements with 2 gm. per cent of bovine albumin at 80° are shown in Fig. 10. In the absence of salt, the viscosity increased sharply, then reached a plateau. Additions of 0.05 M NaCl promoted aggregation of the heated albumin, with a resultant very rapid increase in viscosity. Addition of 0.05 M caproate decreased the rate of viscosity increase, although after 30 minutes the viscosity did rise above that of the sample with no addition, presumably because of the effect of increased ionic strength. Addition of 0.15 M caproate was sufficient to prevent any viscosity increase for a 90 minute period at least. At a temperature of 70°,

the results were similar to those at 80°, except that at the lower temperature 0.05 M caproate was sufficient to prevent any viscosity increase.

Previous observations (14) on the rate of enzyme digestion of albumin solutions with and without added caprylate likewise lead to the conclusion that the caprylate stabilizes the native albumin molecule. This conclusion is also in harmony with the cloud point and nephelometric studies, in that the same visible changes which occur readily in the absence of stabilizers occur after a much longer period in the presence of stabilizing agents. The possibility that the fatty acids might stabilize either native or de-
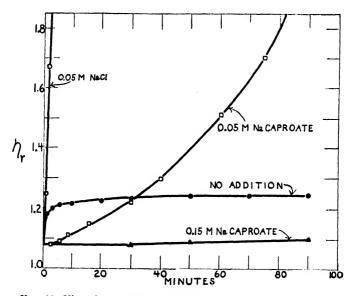
Fig. 10. Viscosity of 2 per cent bovine serum albumin at 80°

natured albumin was previously recognized (1). In view of these later findings, it is probable that the fatty acid salts stabilize the native albumin.

Preliminary Experiments with Other Proteins—It is of obvious interest to determine whether the substances which protect albumin from denaturation also prevent the heat denaturation of other proteins. Preliminary experiments show that this type of stabilization is not a general phenomenon. Cloud point determinations with solutions of approximately 12 gm. per cent of crystalline egg albumin showed an increased cloud point temperature with 0.01 to 0.05 M caprylate. However, unlike that in serum albumin, the increase in cloud point temperature increases with time after mixing the caprylate and egg albumin solutions. The thermal stability of solutions of 10 to 20 gm. per cent of serum γ-globulin, as measured by

the time for gel formation, is decreased by the addition of caprylate or mandelate. Diphtheria toxin, diphtheria antitoxin, and papain are also rendered more heat-labile by caprylate. Further studies with other proteins are planned.

Mode of Action of Stabilizing Substances—The preceding experiments serve to define the properties of compounds that increase the stability of serum albumin against heat denaturation. Any discussion of the mode of action of these compounds is somewhat speculative, because the intra-molecular changes which occur in denaturation are inadequately under-stood.

Measurements of the electrophoretic mobility of albumin-fatty acid systems at constant pH and ionic strength demonstrated that the mobility, and hence in all probability the extent of association, increases with in-crease in the chain length of the fatty acid anion (15). Data discussed in preceding sections of the present paper show that the increased stability is not a function of the increase in net charge on the albumin molecule.

Quantitative measurements of the amount of bound fatty acid anion have been made by ultrafiltration.[1] This quantitative study shows an increase in the amount bound with increase in the chain length of the fatty acid, and shows also that the combination is dependent upon the presence of positively charged groups in the albumin molecule. Fatty acid anions, formaldehyde, and a high concentration of hydroxyl ion all increase the cloud point temperature, and also all react with the positively charged groups of the albumin. Thus it appears probable that certain of the positively charged groups are involved in the stabilization of the heated albumin.

Furthermore, the data show that the degree of stabilization is not de-pendent only on the amount of bound anion. With increase in chain length of the fatty acid, a smaller amount of the fatty acid has to be bound to produce the same stabilizing effect. That this effect does not result primarily from increase in the size of the molecule is evident from data which show that although acetyl tryptophanate is a larger molecule than caprylate it is less effective on the basis of the amount bound. These results stress the importance of both the non-polar portion of the molecule, upon which the magnitude of the effect of a bound molecule depends, and the negatively charged group, which combines with the positively charged groups of the albumin molecule. The non-polar portions of fatty acids are known to associate to form colloidal micelles (16), and it is possible that similar attractive forces may exist between non-polar groups of the albumin molecule and the hydrocarbon chain of the fatty acid. Thus it seems probable that the stabilization is the result of the attraction of differ-ent groups of the albumin molecule for both the polar and non-polar por-

tions of the fatty acid anions.   A tenable conclusion is that this attraction of a single fatty acid molecule for different groups of the protein may aid in preventing the separation of adjacent chains or layers of the albumin molecule, and thus may prevent an "opening out" or extension of the molecule and thereby effect a stabilization against heat denaturation.   Since remarkably low ratios of caprylate to albumin will effect a considerable stabilization, it is apparent that certain selected areas or groups play an essential rôle in the mechanism involved.

SUMMARY

1. Studies have been made of the increased thermal stability of human and bovine serum albumin that results from combination of the albumin with fatty acids and related compounds, by means of "cloud point," nephelometric, and viscometric methods.

2. The protective action of the fatty acid anions, as measured by the cloud point method, increases with increase in chain length of the fatty acids up to 12 carbon atoms when low concentrations are present, but the maximum stabilization at higher concentrations is produced by the C_7 and C_8 acids.

3. Experiments with forty derivatives of carboxylic, sulfonic, sulfuric, and phosphoric acids which contained non-polar groups and with twenty-three compounds of other types lead to the conclusion that the basic requisite structure for stabilizing properties is an anion with a non-polar group attached.   The dissociation constant of the acid derived from the anion is not a primary factor in determining the amount of stabilization.

4. Additions of carboxyl or hydroxyl groups to the non-polar group decrease the effectiveness of a given anion.

5. The protective action of the fatty acid anions is evident at albumin concentrations from 2.5 to 25 gm. per cent, and, in the presence of relatively high concentrations of stabilizing anions, is independent of the ionic strength.

6. The compounds are effective both at neutrality and on the acid side of the isoelectric point of serum albumin.

7. In contrast to the behavior of anions, cations with non-polar groups make the albumin more susceptible to heat denaturation.

8. Nephelometric studies at 50° and 57° demonstrated that low concentrations of the stabilizers are effective for long periods of time.   Both nephelometric and cloud point data showed that remarkably low ratios of fatty acid anions to albumin result in marked stabilization.

9. Viscometric studies showed that the fatty acids prevent viscosity increases in heated solutions.   These results, and previous data on enzyme digestion, demonstrate that the fatty acids stabilize native and not denatured albumin.

10. Preliminary experiments with several other proteins show that this type of stabilization is by no means a general phenomenon.

11. On the basis of these and other studies the possible mode of action of the stabilizers is discussed and it is concluded that the stabilization is the result of association of both the polar and non-polar portions of the anion with certain groups of the albumin molecule in its native state.

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THE COMBINATION OF FATTY ACIDS AND RELATED COMPOUNDS WITH SERUM ALBUMIN

II. STABILIZATION AGAINST UREA AND GUANIDINE DENATURATION

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Serum albumin in common with other proteins is readily denatured by high concentrations of urea or of guanidine salts (1–3). Various inorganic ions have been shown to influence the extent of this denaturation. Greenstein (4) found that the effect of various guanidine salts was greatly influenced by the nature of the anion. Burk (5) has studied in detail the influence of various inorganic electrolytes on the liberation of sulfhydryl groups by urea. Recent experiments which demonstrated that anions with nonpolar groups protect albumin from heat denaturation (6, 7) engendered an interest in the effect of these compounds upon urea denaturation. Studies by Rice *et al.* (8) on the papain digestion of denatured albumin showed that the addition of caprylate to the albumin before the addition of the urea markedly reduced the subsequent rate of enzyme digestion, thus indicating that caprylate had a protective action against urea denaturation. In the present studies, viscosity measurements were used as the criterion of the extent of the denaturation, because denaturation of serum albumin by urea or by guanidine is known to result in a large viscosity increase (1, 2). The results of these experiments have shown that low concentrations of fatty acid salts have a pronounced protective effect against urea denaturation. A preliminary report of these studies has been published recently (9).

EXPERIMENTAL

The serum albumin samples used were the same as those employed in previous studies (6, 7). The γ-globulin was prepared by the plasma fractionation procedure developed by the Department of Physical Chemistry, Harvard Medical School, and obtained by courtesy of Professor E. J. Cohn and the Cutter Laboratories. The urea and the guanidine hydrochloride were recrystallized before use. The various solutions used for the viscosity determinations were at pH 7.5 to 8.0. Viscosity measurements were made at $30° \pm 0.01°$, by use of Ostwald viscometers with water times of 60 to 70 seconds. The viscometers were thoroughly cleaned with dichromate cleaning solution and were dried with the aid of redistilled

acetone after every determination.   The relative viscosities were calculated by comparison of the various solutions containing the albumin with solutions containing no albumin but otherwise of the same composition.

<center>RESULTS AND DISCUSSION</center>

Effect of Caprylate Concentration—The influence of caprylate concentration on the viscosity of serum albumin solutions in 6 M urea is shown in Fig. 1.   These results show clearly that caprylate has a marked protective
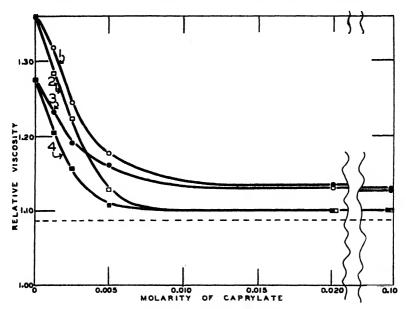
FIG. 1. The influence of caprylate on the urea denaturation of human serum albumin.   All solutions contained 6 M urea, 0.10 M NaCl, and 2 gm. per cent of human serum albumin.   For Curves 1 and 3, the albumin containing the caprylate was added to the urea solution, and the viscosities were determined after 15 minutes and 24 hours respectively.   For Curves 2 and 4, the albumin was added to the urea solution 15 minutes prior to the caprylate, and the viscosities were determined 15 minutes and 24 hours after addition of the caprylate.

action against the urea denaturation of serum albumin.   In these and other similar experiments, when the caprylate was added before the urea, caprylate concentrations of 0.005 M largely prevented the viscosity increase; concentrations of caprylate of 0.010 M were always sufficient for maximum effect in the urea denaturation experiments.   Addition of caprylate after the albumin had been in the urea solution for 15 minutes resulted in a partial return of the viscosity to that of the solution in which the caprylate was added prior to the urea.   Data to be presented later

(Fig. 4) show that the amount of this "regeneration" is dependent upon the time of exposure of the albumin to the high urea concentration.

The action of the caprylate was complete within a short period after its addition. As is shown in Fig. 1, when caprylate was present in sufficient amount for maximum effect, little or no viscosity change occurred between the 15 minute and 24 hour measurements. When the caprylate was not present in a concentration sufficient for maximum effect, a further viscosity increase, characteristic of the urea denaturation in the absence of caprylate, occurred between the 15 minute and the 24 hour measurements.
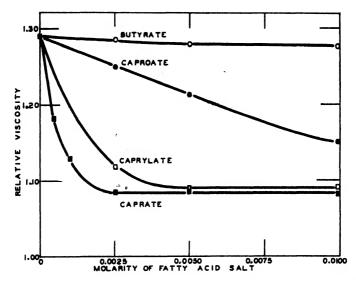
Fig. 2. The influence of various fatty acid anions on the urea denaturation of bovine serum albumin. All solutions contained 6 M urea, 0.10 M NaCl, and 2 gm. per cent of crystalline bovine albumin. Viscosity measurements were made 24 hours after addition of the fatty acid and the albumin to the urea.

Influence of Other Fatty Acid Salts—The thermal stabilization of albumin by fatty acid salts increases with the chain length of the fatty acid (6, 7). A similar effect of chain length was found in urea denaturation experiments, the results of which are shown in Fig. 2. When caprylate or caprate was present in concentrations sufficient for maximum effect, the viscosity in the presence of caprate was slightly lower than that in the presence of caprylate. This small difference may have been due to a slightly decreased extension of the molecule, or to a decreased solvation of the albumin molecule in the presence of the caprate, or to a combination of these two effects.

The variation in stabilization with increase in chain length is greater than the variation in the amount of fatty acid anion bound to the albumin,

as measured by ultrafiltration.[1]   For example, 0.0015 M caprylate had about the same effect in these viscosity experiments as 0.010 M caproate.   However, at these concentrations there were about 2 to 3 times as much caproate bound.   Similarly, on a concentration basis about 1.5 times more bound caprylate than bound caprate were required to produce the same effect.   These results are comparable to similar relationships noted in heat denaturation studies (7).
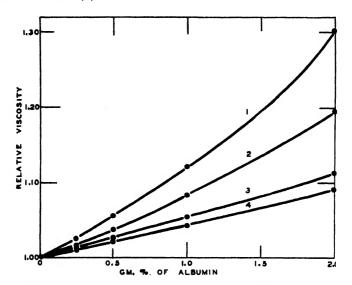
Fig. 3. Relative viscosities of solutions containing different concentrations of human serum albumin in water and 6 M urea.   All solutions contained 0.10 M NaCl. Curve 1, albumin in 6 M urea for 2 hours; Curve 2, albumin in 6 M urea for 2 hours, and then sodium caprylate was added to give 0.020 M caprylate; Curve 3, albumin and 0.020 M caprylate in 6 M urea for 2 hours (caprylate and albumin mixed before addition to the urea); Curve 4, albumin in water.

Variation in Albumin Concentration—In order to determine the weight intrinsic viscosities of the urea-denatured albumin under different conditions, viscosity measurements were made at albumin concentrations of 0 to 2 gm. per cent.   The results of these measurements are shown in Fig. 3.   The high weight intrinsic viscosity of albumin noted in the urea solution (11.2) is attributable to an unfolding of the molecule with the consequent increase in axial ratio (2, 10, 11).   That the viscosity changes are not due to aggregation is evident, since albumin remains monodisperse and, in addition, does not change in molecular weight when denatured by urea (12).

[1] Boyer, P. D., Ballou, G. A., and Luck, J. M., unpublished.

The small difference in the weight intrinsic viscosities of the albumin in water and the albumin in 6 M urea containing 0.02 M caprylate (4.4 and 5.2 respectively) can be attributed either to a slight extension of the molecule or to a solvation of the molecule by about 0.18 to 0.27 gm. of urea per gm. of albumin.   If this amount of solvation is assumed, then it may be concluded that the caprylate prevents any unfolding of the native molecule.

Variation in Time of Caprylate Addition—As noted above, addition of caprylate to albumin solutions after denaturation by urea resulted in a
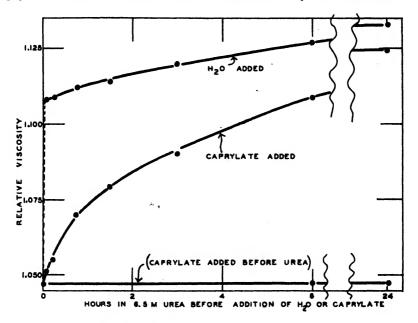
FIG. 4. Viscosity decrease produced by caprylate after exposure of albumin to urea for various time intervals.   To solutions of human serum albumin in 6.5 M urea, sufficient water or sodium caprylate solution was added at various time intervals to give a solution containing 1.0 gm. per cent of albumin, 0.10 M NaCl, with or without 0.020 M caprylate.

decrease in the viscosity of the solution.   The effect of addition of either water or a dilute solution of sodium caprylate to albumin at various intervals after the addition of the albumin to the urea is shown in Fig. 4. The initial marked increase in viscosity noted in the absence of caprylate occurred very rapidly.   This initial high viscosity was evident even when measurements were made as soon as possible after addition of the albumin. This initial rapid rise was followed by a more gradual, less pronounced, viscosity increase during the next 24 hours.   When caprylate was added after the albumin had been in the urea solution for 5 minutes, 92 per cent

of the increase in the relative viscosity was removed by the caprylate. Increase in the time of exposure of the albumin to the urea resulted in a progressive decrease in the effectiveness of the caprylate. After 24 hours only 13 per cent of the viscosity increase was removed by addition of caprylate. These results offer an explanation for the observed differences in rate of enzyme digestion of urea-treated serum albumin when the caprylate was added with the albumin or 24 hours after the albumin.

When the caprylate was added as soon as possible after the addition of the albumin, or if the caprylate was added to the urea solution before the albumin, the resulting viscosity was the same as when the caprylate and the albumin were mixed before addition to the urea. These data indicate that the combination of the caprylate with the albumin, which enables the albumin to resist urea denaturation, takes place very rapidly.

The above results demonstrate that the urea denaturation probably occurs in two stages or steps. The observations of Lundgren and Williams (13, 14) are clearly relevant. The initial change or unfolding of the molecule is very rapid, and the extended molecule which results can be refolded to a large extent by the addition of caprylate. A second much slower change takes place, which modifies the molecule in such a manner that addition of caprylate results in only a small decrease in viscosity. Whether the albumin molecules, refolded by the prompt addition of caprylate, have the same spatial configuration as the native molecules cannot be adduced from the present data. Likewise, further studies are necessary to ascertain whether the molecule refolded by the addition of caprylate immediately after the addition of the albumin has the same physical properties as the original molecule. When the albumin is "regenerated" by removal of the urea, the resulting molecule apparently has slightly different physical properties.

Denaturation of γ-Globulin by Urea—Solutions of serum globulin, like those of albumin, exhibit a marked viscosity increase when the globulin is denatured by urea (15). In contrast to the behavior of the albumin, solutions of 2 gm. per cent of γ-globulin in 6 M urea were not protected from denaturation by the presence of 0.0025 to 0.10 M caprylate. This observation is in harmony with the lack of effect of caprylate on the heat coagulation of γ-globulin (7).

Influence of Caprylate on Denaturation by Guanidine—The denaturation of serum albumin by guanidine hydrochloride was not as readily prevented by caprylate as was the urea denaturation. A 6 M guanidine hydrochloride solution produced a much greater viscosity increase than did a comparable urea solution. These results are in accordance with earlier observations that guanidine hydrochloride is a more powerful denaturing agent than urea (10). When 6 M guanidine hydrochloride solutions were used, capryl-

ate in concentrations up to 0.10 M had no protective action. As the guanidine concentration was decreased, the effect of caprylate became manifest. A 2.5 M guanidine hydrochloride solution produced about the same viscosity increase as 6 M urea, and at this guanidine concentration caprylate had a pronounced stabilizing effect, as is shown by the data given in Fig. 5. These results differ from those obtained with urea in that relatively high concentrations of caprylate were less effective than lower concentrations, an observation that is not readily explainable, and also in that the
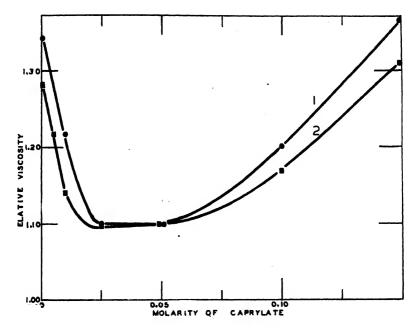
Fig. 5. The influence of caprylate on the denaturation of human serum albumin by guanidine hydrochloride. All solutions contained 2.5 M guanidine hydrochloride and 2 gm. per cent of albumin. Curves 1 and 2 represent respectively the relative viscosities 24 hours and 15 minutes after addition of the albumin.

concentration of caprylate necessary for maximum effect was greater in the guanidine experiments.

Comparison of Action of Caprylate and Sulfate—This comparison was made because Burk found sulfate to be one of the most effective inorganic ions for the prevention of urea denaturation (5), and Greenstein noted that guanidine sulfate had less denaturing effect than other guanidine salts (4). The results of measurements of the viscosity of solutions containing 2 gm. per cent of albumin, 2.5 M guanidine hydrochloride, and variable amounts of sodium sulfate are presented in Fig. 6. These results

show that the sodium sulfate had a protective effect against the viscosity increase otherwise produced, but that the amount of sulfate required for maximum effect was over 20 times as great as the amount of caprylate required.

Mode of Action of Stabilizing Substances—Only relatively low concentrations of caprylate are necessary to protect serum albumin against urea denaturation. In addition, as measured by ultrafiltration studies, only part of the caprylate present is bound to the albumin.[1] The effectiveness
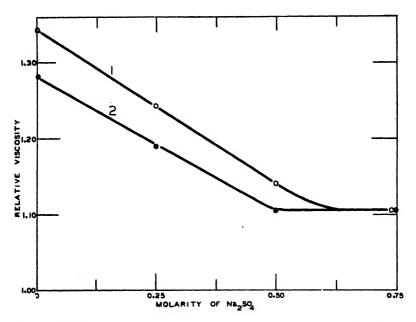
FIG. 6. The influence of sodium sulfate on the denaturation of human serum albumin by guanidine hydrochloride. All solutions contained 2.5 M guanidine hydrochloride and 2 gm. per cent of albumin. Curves 1 and 2 represent respectively the relative viscosities 24 hours and 15 minutes after addition of the albumin.

of such low concentrations demonstrates that, as with heat denaturation, combination of the fatty acid anions with certain specific groups or areas of the albumin molecule renders the molecule more resistant to urea denaturation. As previously mentioned (9), the effectiveness of small concentrations of caprylate against both heat and urea denaturation suggests that caprylate inhibits the same initial changes in both types of denaturation. Subsequent changes are obviously not the same, since the heated albumin aggregates, while the urea-denatured albumin remains monodisperse. The possible mechanisms of action, discussed in the paper on

heat denaturation (7), are probably also applicable to the experiments on urea denaturation.

If the mechanism of action of the sulfate ions·and of the fatty acid anions is the same, then the effect probably depends only upon attraction for or association with certain positive groups of the albumin (16). However, since with increase in chain length of the fatty acid a smaller amount of bound fatty acid is necessary to produce a given effect, it is apparent that the non-polar portion of the fatty acid anion must also have some function in the stabilization against urea denaturation.

SUMMARY

1. Studies have been made of the influence of fatty acid salts on the denaturation of human and bovine serum albumin by urea or guanidine hydrochloride, viscosity increase being used as the criterion of denaturation.

2. Low concentrations of fatty acid salts prevented the viscosity increase which otherwise resulted when the albumin was dissolved in 6 M urea.

3. The effect of the fatty acid anions increased with the chain length, and the increase in stabilizing properties was greater than the increase in the amount of bound fatty acid.

4. Concentrations of fatty acid salts sufficient for maximum effect resulted in a weight intrinsic viscosity for the albumin slightly greater than that of the albumin dissolved in water. This difference may have been due to a slight extension of the molecule in the presence of the urea, or to a solvation of the albumin by the urea, or to a combination of these effects.

5. Addition of caprylate to albumin solutions previously denatured by 6 M urea resulted in a prompt, pronounced viscosity decrease. The extent of this refolding of the extended molecule decreased with the time of exposure to the urea. These results indicate that the denaturation of the serum albumin by caprylate occurs in two stages, and that the initial rapid unfolding which occurs is readily prevented or reversed by caprylate.

6. In contrast to the behavior of serum albumin, serum γ-globulin was not protected from urea denaturation by the addition of caprylate.

7. Caprylate also prevented the viscosity increase which otherwise occurred when the albumin was dissolved in 2.5 M guanidine hydrochloride, but had no stabilizing effect when 6 M guanidine hydrochloride was used. Sodium sulfate likewise prevented the denaturation by 2.5 M guanidine hydrochloride, but when compared on a concentration basis was roughly only about one-twentieth as effective as caprylate.

8. The data indicate that the action of the fatty acid anions is due to their combination with certain groups or areas of the albumin molecule,

and that the effect is probably the result of combination of the anion with both the positive groups and the non-polar portions of the albumin.

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THE EFFECT OF RIBOFLAVIN DEFICIENCY UPON CARBOHYDRATE METABOLISM IN ANOXIA

BY MARY E. WICKSON AND AGNES FAY MORGAN

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It has been reported by many investigators that a normal fasted animal exposed to low oxygen tension will increase its carbohydrate stores over the fasting values at sea level (1–6). The source of this increased carbohydrate appears to be protein, and the stimulus to glyconeogenesis may be through the adrenal cortex or the pituitary gland.

Riboflavin has been shown to be related to both carbohydrate (7, 8) and protein (9–12) metabolism, and there is conflicting evidence that the adrenal cortex may or may not be related to the phosphorylation of riboflavin (13–15). This substance, because of its relation to carbohydrate and protein metabolism, may be a necessary component of the carbohydrate or protein systems that are stimulated by low oxygen tension. Thus, a riboflavin-deficient animal might be unable to respond to anoxic anoxia as a normal animal does; that is, by increasing its total carbohydrate levels. If the deficient animals do not respond normally to low oxygen tension, this failure might be due not only to the derangement of the oxidase system but also to the diminished food intake which accompanies the deficiency, or to some permanent structural change brought about by the deficiency state.

The problem of the relation of riboflavin to the carbohydrate metabolism that is stimulated by low oxygen tension was studied by comparing the tissue carbohydrate levels in normal, riboflavin-deficient, and pair-fed rats held at atmospheric and reduced pressure for a 24 hour test period.

EXPERIMENTAL

Male and female rats of the Long-Evans-Wistar strain, weighing 37 to 45 gm., were placed on a riboflavin-deficient diet for a period of from 4 to 6 weeks, or until their depletion was indicated by a weight plateau for 4 to 5 days or a weight loss of 5 to 6 gm. The composition of the diet was vitamin-free (Labco) casein 22.0, sucrose 66.5, hydrogenated cottonseed oil 9.0, salts (16) 2.5. The vitamin supplements in mg. per rat per day were thiamine hydrochloride and pyridoxine hydrochloride, each 0.02, calcium pantothenate and *p*-aminobenzoic acid, each 0.1, and choline and inositol, each 5.0. Vitamin A (143 I.U. daily) was supplied from gray-fish oil, and vitamin D (10 I.U. daily) as irradiated ergosterol. 1 ml. of

rice bran concentrate[1] extracted with fullers' earth (1 : 10 dilution) was given three times a week.[2]

Three groups of rats were set up after the depletion period, and these groups further divided just prior to the 24 hour test period. These were (1) normal, receiving 0.01 mg. of riboflavin daily, (2) pair-fed, receiving the same amount of riboflavin but restricted to the quantity of basal diet eaten by the deficient rats, and (3) those receiving no riboflavin. The animals were maintained for 7 to 9 weeks after depletion on these diets.

The test period consisted of a 24 hour fast at reduced or atmospheric pressure, after which the animals were killed and their liver glycogen, muscle glycogen, blood sugar, and liver riboflavin determined. During the 24 hour test period the rats received no food or water, these being removed at a uniform time before the exposure period. Sea level control groups were kept under the same conditions as the low pressure groups, except for the oxygen tension. During the anoxic period all animals were confined in individual, reclining, wide mouth glass jars, which had strips of wire screening for platforms, and which were sealed with canning lids and screw tops through which passed two metal tubes, one connecting with the air line and the other with the vacuum system. The jars were supported on a rack, and each series of jars was connected with a separate air and vacuum line. The pressure inside the jars was controlled by means of a needle valve inserted in the air and vacuum lines.[3] The pressure used for these experiments was 349 mm. of Hg corresponding to 20,000 ft. of elevation, and an adequate air flow was provided. The pressure was lowered at the rate of 3000 ft. per minute. The temperature of the chamber in which the animals were kept was 23–24° and care was taken to maintain this temperature in the room in which the animals were sacrificed.

The riboflavin-deficient animals were divided into two groups just prior to the anoxia period. Each rat in one of these groups received an intraperitoneal injection of 1 ml. of 0.9 per cent NaCl solution containing 0.1 mg. of riboflavin, just before being placed in the chamber. All the other deficient animals were injected with 1 ml. of 0.9 per cent NaCl by the same route.

The animals sacrificed were anesthetized with sodium amytal by intraperitoneal injection (140 mg. per kilo) and were unconscious within 3 to 4 minutes.

[1] This was the concentrate marketed under the name of Galen "B" by the Galen Company, Inc., Berkeley, California.

[2] The growth of animals during the first 2 weeks after weaning was 0.7 gm. daily per rat with the concentrate and 0.5 gm. daily without it. This additional growth may have been due to the slight amount of riboflavin that was present in the concentrate (0.15 γ per ml. as fed).

[3] The apparatus was designed by V. V. Herring, Institute of Experimental Biology, University of California, Berkeley.

The left gastrocnemius was removed immediately and placed in cold 30 per cent KOH for the determination of glycogen. The abdominal cavity was opened and blood removed from the inferior vena cava. Blood proteins were precipitated immediately with $CuSO_4$ and Na_2WO_4 (17). The liver was then removed and placed in cold 30 per cent KOH in 50 ml. Pyrex centrifuge tubes, the tubes covered with tin-foil, placed immediately in a boiling water bath, and left there for about 1 hour, after which they were cooled and weighed. The tubes containing muscle samples were weighed and then placed in the boiling water bath for 1 hour. To precipitate the glycogen 1 volume of water and 2 volumes of 95 per cent ethyl alcohol were added, the tubes were placed in the boiling water bath until the boiling point was reached, and were removed and replaced in the bath the second time. The glycogen tubes were then stored overnight in the ice box. The next morning the glycogen was centrifuged, the supernatant liquid poured off, 1 N H_2SO_4 added, the tubes covered again with tin-foil, and the 2 to 3 hour hydrolysis begun. After hydrolysis the tubes were stored overnight in the ice box. For the glucose determinations the contents of the tubes were transferred quantitatively to volumetric flasks, neutralized with 5 per cent KOH (phenol red), and made to volume. 2 ml. aliquots were taken for the determination of reducing sugar.

Glucose was determined by the method of Benedict (18). The color reagent used was prepared by a slight modification of that described by Benedict (19) and all colorimetric readings were made on a Klett-Summerson colorimeter.

Liver riboflavin was determined by the microbiological method of Snell and Strong (20). Extraction of tissue riboflavin was accomplished by autoclaving the finely ground tissue in 0.04 N H_2SO_4 at 15 pounds pressure for $\frac{1}{2}$ hour, followed by digestion at pH 4.5 with 0.5 per cent clarase for 2 hours at 50°.

Results

The data reported in this paper were obtained on rats fed a basal diet containing Labco casein, which contained 0.35 to 0.40 γ of riboflavin per gm. as determined by microbiological assay.[4] The total amount of riboflavin that a rat ingesting 10 gm. of the diet would receive was about 0.9 γ, since in addition to the riboflavin of the basal diet the fullers' earth-extracted rice bran concentrate contained 0.15 γ per ml. The rats exhibited severe riboflavin deficiency symptoms, spectacled eyes, general alopecia, and corneal opacities.

The four groups of carefully selected rats, matched as to sex, weight, and

[4] The method adopted for preparation of the casein for assay included autoclaving $\frac{1}{2}$ hour at 15 pounds pressure in 0.5 N H_2SO_4, addition of solid NaOH until flocculation occurred (about pH 2.4), and filtration, followed by neutralization of the filtrate.

litter, were designated as follows: (1) normal, those which had received the diet *ad libitum* and 0.01 mg. of riboflavin daily; (2) deficient, those which had received the diet *ad libitum* but no riboflavin; (3) injected deficient, those treated identically with the deficient group but which had received one injection of 0.1 mg. of riboflavin immediately before entering the low pressure chambers; (4) pair-fed, those which had received only the amount of diet eaten by the deficient group but 0.01 mg. of riboflavin daily.

All groups lost slightly more weight at low pressure than at sea level during the 24 hour fast, but the difference was significant only in the normal group. Deficient and injected deficient animals lost more weight at sea level than did the normal animals, but not more than did the pair-fed animals (Table I).

TABLE I

Average Weight, Daily Food Intake, and Weight Loss during 24 Hour Fast of Normal and Riboflavin-Deficient Rats

Experimental group		No. of animals	Average weight	Daily food intake	Weight loss
			gm.[*]	*gm.*	*per cent body weight*
Normal	Sea level	9	196	9	5.5 ± 0.4
	20,000 ft.	8	203	9	9.2 ± 0.8
Deficient	Sea level	8	88	5	9.3 ± 1.2
	20,000 ft.	10	87	5	11.0 ± 0.9
Injected deficient	Sea level	8	87	5	9.3 ± 1.0
	20,000 ft.	9	90	5	11.5 ± 0.8
Pair-fed	Sea level	8	117	5	7.0 ± 1.2
	20,000 ft.	8	120	5	8.8 ± 0.4

[*] Age, 14 to 18 weeks.

Liver Riboflavin—The liver riboflavin of the deficient rats was lower than that of the normal, pair-fed, and injected deficient groups. The injected rats had higher liver riboflavin than did the deficient group, evidently being able to store some of the injected riboflavin. There was no difference between sea level and low pressure groups, except in the pair-fed animals, which had slightly lower liver riboflavin at reduced than at atmospheric pressure (Fig. 1). The normal group also exhibited this tendency.

Blood Sugar—Riboflavin deficiency produced lower than normal fasting blood sugar levels (Table II, Fig. 2). This has also been reported by Axelrod, Lipton, and Elvehjem (21) in riboflavin-deficient dogs. The low blood sugar was apparently not due to inanition, as the pair-fed controls had normal levels. The injection of one dose of 0.1 mg. of riboflavin
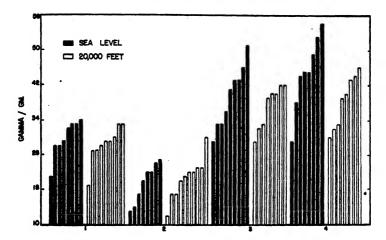
FIG. 1. Riboflavin content of livers of normal and riboflavin-deficient rats at sea level and exposed to anoxia (20,000 ft. altitude) for 24 hours. Each bar represents the liver riboflavin of a single animal. Group 1, riboflavin-deficient but injected with 0.01 mg. of riboflavin just before exposure; Group 2, riboflavin-deficient; Group 3, normal, fed *ad libitum;* Group 4, normal but restricted to food intake of the deficient group.

TABLE II

Effect of Anoxic Anoxia on Carbohydrate Levels of Fasting Normal and Riboflavin-Deficient Rats

Experimental group		Liver glycogen		Muscle glycogen		Blood sugar		Liver riboflavin	
		No. of animals		No. of animals		No. of animals		No. of animals	
			mg. per cent		*mg. per cent*		*mg. per cent*		*γ per gm.*
Normal	Sea level	5 ♀	158 ± 51	5 ♀	540 ± 36	5 ♀	115 ± 7	9	39 ± 2
		4 ♂		3 ♂		4 ♂			
	20,000 ft.	3 ♀	2081 ± 322	3 ♀	580 ± 87	4 ♀	148 ± 8	8	37 ± 2
		3 ♂		4 ♂		4 ♂			
Deficient	Sea level	6 ♀	73 ± 15	6 ♀	454 ± 40	6 ♀	56 ± 6	8	19 ± 2
		2 ♂		1 ♂		2 ♂			
	20,000 ft.	5 ♀	208 ± 96	6 ♀	474 ± 54	6 ♀	103 ± 22	10	21 ± 2
		3 ♂		3 ♂		4 ♂			
Injected deficient	Sea level	6 ♀	165 ± 56	5 ♀	473 ± 38	5 ♀	58 ± 6	8	29 ± 2
		2 ♂		2 ♂		2 ♂			
	20,000 ft.	5 ♀	1318 ± 224	3 ♀	593 ± 43	5 ♀	164 ± 25	9	28 ± 1
		3 ♂		5 ♂		4 ♂			
Pair-fed	Sea level	5 ♀	241 ± 54	4 ♀	482 ± 40	5 ♀	103 ± 6	8	45 ± 3
		3 ♂		3 ♂		3 ♂			
	20,000 ft.	4 ♀	2753 ± 356	3 ♀	663 ± 25	4 ♀	137 ± 11	9	38 ± 2
		4 ♂		4 ♂		4 ♂			

into the deficient rats did not raise the blood sugar level at atmospheric pressure.

Liver Glycogen—It cannot be determined definitely from these data whether riboflavin deficiency affected the sea level liver glycogen values,
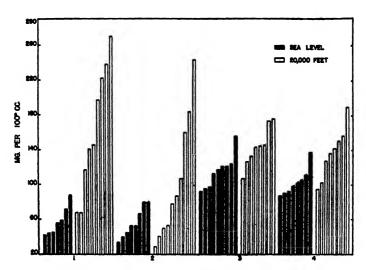
FIG. 2. Fasting blood sugar levels of normal and riboflavin-deficient rats at sea level and exposed to anoxia (20,000 ft. altitude) for 24 hours. The groups are as indicated in Fig. 1.

TABLE III

Effect of Anoxia (349 Mm. of Hg for 24 Hours) on Carbohydrate Levels of Normal and Riboflavin-Deficient Rats; Increase over Sea Level Values*

Group	Liver glycogen	$\frac{D.}{S.E.}$	Muscle glycogen	$\frac{D.}{S.E.}$	Blood sugar	$\frac{D.}{S.E.}$
	mg. per cent		*mg. per cent*		*mg. per cent*	
Normal.................	1923 ± 316	6.1	40 ± 87	0.4	33 ± 10	3.3
Deficient...............	135 ± 97	1.4	20 ± 66	0.3	47 ± 23	2.0
Injected deficient.......	1216 ± 230	5.3	156 ± 62	2.5	106 ± 25	4.2
Pair-fed................	2512 ± 359	7.0	181 ± 47	3.8	34 ± 12	2.8

* $\frac{D.}{S.E.}$ = (difference of means)/(standard error of the difference). A value of 2 or more was taken as indicating a significant difference.

but Table II and Fig. 3 show the tendency of the deficient animals to have lower liver glycogen than any other group.

The normal animals exhibited a typical increase in liver glycogen when exposed to low oxygen tension (Tables II and III, Fig. 3). The riboflavin-

deficient rats were not capable of bringing about this increase in liver glycogen at reduced pressure, although some of the animals raised their liver glycogen well above the sea level average for their group. This inability of the deficient animals to increase the store of liver glycogen was not due to inanition, however, because the pair-fed rats evinced better glyconeogenesis than did the normal rats. The failure of the deficient
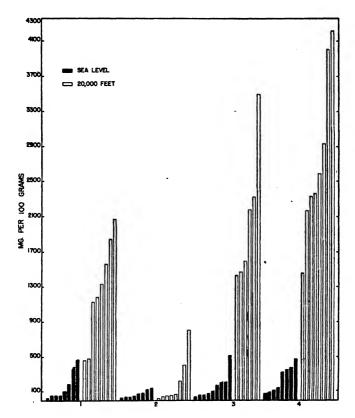
Fig. 3. Liver glycogen of fasting normal and riboflavin-deficient rats at sea level and exposed to anoxia (20,000 ft. altitude) for 24 hours. The groups are as indicated in Fig. 1.

rats to increase their liver glycogen was apparently a functional disability and not due to any structural change brought about by the deficiency, because when riboflavin was injected into the deficient animals they were nearly as capable of glyconeogenesis as were the normal rats.

Muscle Glycogen—The muscle glycogen of all groups at sea level was similar (Table II, Fig. 4), with some tendency for the normal group to show

greater values than the others. The normal and deficient groups did not significantly increase their muscle glycogen when exposed to reduced pressure, but the pair-fed and injected deficient rats made small but apparently significant increases in muscle glycogen under these circumstances.
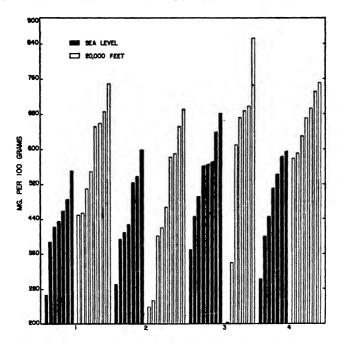
FIG. 4. Muscle glycogen of normal and riboflavin-deficient fasting rats at sea level and exposed to anoxia (20,000 ft. altitude) for 24 hours. The groups are as indicated in Fig. 1.

DISCUSSION

The normal fasting animals subjected to reduced pressure for 24 hours increased their liver glycogen and blood sugar significantly when compared with sea level controls. This has been reported by Evans (1), by Lewis, Thorn, Koepf, and Dorrance (2), by Long, Katzin, and Fry (3), and by Langley and Clarke (4). The normal animals did not show significant increases in muscle glycogen when exposed to low oxygen tension. Evans (1) and Lewis and coworkers (2) obtained higher muscle glycogen in normal animals subjected to low oxygen tension, but Long, Katzin, and Fry (3) reported no difference in this respect between normal animals and those treated with adrenocortical extract, a condition which produced effects like those of anoxia.

The state of riboflavin deficiency produced a lower than normal fast-

ing blood sugar, which was not due to inanition and was not affected by the injection of 0.1 mg. of riboflavin just previous to the beginning of the 24 hour fast. The liver glycogen levels of the fasting riboflavin-deficient rats at sea level were somewhat lower than those of the normal, pair-fed, and injected deficient animals, but the variation within groups was too great to make the difference significant (Fig. 3). The muscle glycogen of the deficient rats was also at a slightly lower level than normal at sea level (Fig. 4). It appears from these data that riboflavin may be concerned with glucose formation at sea level under fasting conditions.

When the riboflavin-deficient animals were exposed to low oxygen tension in the fasting state, they were incapable of stimulating glyconeogenesis at a normal rate. Although there was much variation, many of them (Fig. 2) increased their blood sugar to higher than sea level values, but even those animals with high blood glucose failed to increase the liver glycogen to the usual level seen under reduced pressure. The muscle glycogen of the deficient animals did not increase at low pressure, but this was also true of the normal animals. These data indicate that the ribo-flavin deficiency state rendered the animals incapable of increasing their carbohydrate levels at reduced pressure, probably due primarily to failure of increased formation of glucose, or to inadequate glycogenesis or to both. To determine the true cause, glucose excretion of fasting animals under anoxia should be determined, as well as the liver glycogen levels of ribo-flavin-deficient rats given a glucose meal just before exposure to reduced pressure.

The injection of riboflavin into the deficient animals apparently had no significant effect upon the carbohydrate levels at atmospheric pressure. At reduced pressure, however, the effect of the single injection was immediate and quite marked. The blood glucose rose to an average higher than that of any other group[5] and liver glycogen was deposited to nearly the same degree as in the normal rats. This group of deficient animals was able to respond to low oxygen tension, in one respect more effectively than the normal animals, that is, by greater rise in blood glucose. This seemed to indicate specificity of function of riboflavin in the glyconeogenic mecha-nism. The increase in liver glycogen was less than that of the normal and pair-fed groups but the increase in muscle glycogen was greater (Table III). Possibly the liver glycogen level might have been increased further if the riboflavin had been administered in two or more smaller doses during the anoxic period, to prevent loss by excretion of temporary excess.

[5] Statistical analysis does not show a significant difference between the blood sugar increase of the deficient and injected deficient rats, because the standard deviation is large. However, the injected animals definitely increased their liver glycogen, which probably indicated higher blood glucose levels.

The carbohydrate levels of the pair-fed rats were the same as those of the normal at sea level, but under anoxia the increase in both muscle and liver glycogen appeared to be greater than was seen in the normal group. The final levels achieved were not different in the muscle glycogen but appeared to be significantly higher in the liver glycogen of the pair-fed group. This effect of chronic partial inanition was not unexpected, but it apparently occurred only in the presence of riboflavin, since the deficient group achieved practically no increase in glycogen.

If the presence of riboflavin is necessary for normal performance by the carbohydrate-forming mechanism stimulated by low oxygen tension, it might be expected that the riboflavin content of the tissues would be changed during exposure to anoxia. The riboflavin content of the livers varied with the riboflavin intake of the animal (Fig. 1). This has been reported by Fraser, Topping, and Isbell (22). Under anoxia the only significant change was in the pair-fed group, in which there was a slight decrease. It cannot be determined whether this decrease represented a destruction of riboflavin at low pressure or a mobilization of riboflavin in some other tissue at the expense of the liver. Govier (23) recently reported the work of Grieg, who found during exposure of animals to shock a breakdown of alloxazine adenine dinucleotide, which was resynthesized when riboflavin was administered. It may be significant that the greatest amount of glyconeogenesis occurred in our pair-fed group in which the riboflavin store in the liver was reduced.

There was a suggestive correlation between the liver stores of riboflavin and the increases in liver glycogen produced under anoxia. Thus the livers of the pair-fed rats had the most riboflavin and made the greatest increase in glycogen, the normal livers were next in concentration of both, then those of the injected deficient, and finally of the deficient group. Apparently the liver riboflavin threshold value required for glycogenesis was attained by the injected deficient but not by the deficient group. This riboflavin concentration of the liver may be an accompanying or following value, however, rather than the conditioning factor of the glycogenesis.

Hailman (24) reported recently the effect of riboflavin in preventing the depression of the linguo-maxillary reflex in the dog, and suppression of the contraction of the smooth muscle of the frog esophagus, caused by anoxia. He believed the effect of the riboflavin was due to improved cellular oxidation.

The riboflavin-deficient animals had two characteristics which might indicate adrenal cortical insufficiency. The fasting blood sugar was low at sea level, and the carbohydrate mechanism under an anoxic condition appeared to be handicapped. One might consider the interrelation of symptoms of the two conditions in terms of the theory of Verzár and Laszt

(13) that the adrenal cortex is necessary for the phosphorylation of riboflavin. If this were so, a riboflavin-deficient animal injected with riboflavin hydrochloride could not utilize it to the fullest extent if the rat were suffering from adrenal insufficiency as a result of the prolonged deficiency. Since the injected animals were capable of utilizing unphosphorylated riboflavin, it must be concluded either that the deficiency produces no adrenal cortical insufficiency, or that the adrenal cortex is not necessary for the phosphorylation of riboflavin. There is more evidence in favor of the latter conclusion, since the deficient animals reacted under anoxia, so far as their carbohydrate metabolism was concerned, as do adrenalectomized rats (1–6). The failure of the carbohydrate mechanism was not as complete in the deficient group as has been reported for adrenalectomized animals, but this may be due to incompleteness of the riboflavin deficiency.

SUMMARY

1. Rats deficient in riboflavin could not increase their liver glycogen levels, when exposed to low oxygen tension, to the same degree as normal animals. This inefficiency was not due to the lower food intake of the deficient animals, nor to any permanent structural change.

· 2. Injection of riboflavin into the deficient animals just before the exposure period began permitted normal response to low oxygen tension.

3. The deficient animals had lower blood glucose when fasted at sea level than did normal animals, a condition not seen in the pair-fed normal group.

4. The riboflavin content of the rat livers varied with the riboflavin intake.

Riboflavin is indispensable for the operation of the glyconeogenic mechanism that is stimulated by anoxic anoxia, and may be involved in both glucose and glycogen formation.

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NICOTINIC ACID VALUES BY CHEMICAL AND MICROBIO-
LOGICAL METHODS. EFFECT OF HYDROGEN PEROXIDE
AND INFRA-RED RAYS ON NICOTINIC ACID

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For comparative purposes the nicotinic acid content of various products
has been determined by both chemical and microbiological methods.
Along with this comparative study, work has been done on treatments of
the extracts and a study of the effects produced as a result of the treat-
ment on the nicotinic acid content previously determined. A survey of
the literature discloses that numerous discrepancies in values are obtained
for nicotinic acid dependent on the material analyzed, the method used,
and the treatment given the material prior to the determination. Not
only do these values differ when the determinations are carried out by
chemical and microbiological methods, but they also vary according to
the products analyzed, indicating that certain types of materials are en-
countered that affect the determination when carried out by one method
on some products that do not interfere in other procedures.

The microbiological method selected for use in this work is the proce-
dure of Barton-Wright (1) which we find possesses advantages over other
microbiological procedures we have tried in this laboratory. This method
differs from that of Krehl *et al.* (2) in the following points: (*a*) the casein
hydrolysate is doubled in concentration, (*b*) the concentration of biotin
is raised to 0.4 γ per liter, (*c*) xanthine and xylose are added to the basal
medium, (*d*) synthetic *dl*-tryptophane is used instead of natural *l*-trypto-
phane to avoid possible nicotinic acid contamination, and (*e*) the commer-
cially available vitamin-free casein hydrolysate, Smaco, is used instead of
preparing the hydrolysate. This hydrolysate proved free of niacin and
satisfactory in this work.

The chemical procedures employed were the methods worked out by a
group of cereal chemists under the chairmanship of Mr. Steele (3), and a
modification of the Bina, Thomas, and Brown (4) procedure in which the
samples were treated and run separately by that method. Aliquots of
the samples prepared by the collaborative method were used for assay
by the microbiological procedure.

Brown, Thomas, and Bina (5) found that cereal extracts contained two
types of compounds that are measured as nicotinic acid and are differen-
tiated chemically by the fact that one is readily oxidized and loses its

properties as a chromogen, while the second type is stable to hydrogen peroxide oxidation and continues to be reactive in the oxidized extract. These authors presented the results of analyses made before and after the treatment of cereal extracts with oxidizing reagents, and found that oxidation shows the presence of interfering substances that affect the values obtained for nicotinic acid by the microbiological method corresponding to similar values with the chemical method. Since Krehl, Strong, and Elvehjem (2) reported that they obtained no difference in results when they treated their extracts with hydrogen peroxide, further experiments were made with this reagent and the results obtained confirm our previous findings. Not only do we find this true on the products reported in that paper but also on wheat bran and malt extracts treated similarly to whole wheat. The value for wheat bran dropped from 215 to 154 and for barley malt from 71 to 52 γ per gm. as a result of the oxidation with hydrogen peroxide and microbiological determination.

In our previous publication we found no destruction of pure nicotinic acid when samples were treated with hydrogen peroxide, and complete recovery was obtained regardless of whether the chemical or microbiological method was used. These results are also confirmed in this work and show that hydrogen peroxide does not oxidize nicotinic acid under the conditions used and that the reduction in values obtained for nicotinic acid in certain products by this treatment is due to the destruction of interfering substances contained in the extracts.

In the experimental part of this paper we find that infra-red radiation does not destroy pure nicotinic acid when solutions are evaporated by means of an infra-red lamp and that the nicotinic acid is recovered completely after the treatment. However, when a solution of crystalline nicotinic acid is treated with hydrogen peroxide and evaporated to dryness by means of infra-red radiation, the nicotinic acid is almost completely destroyed. This method of destruction of nicotinic acid was not expected and is being studied further in our laboratory to determine the mechanism of the reaction and the products produced.

EXPERIMENTAL

Preparation of Extracts—Two methods of extract preparation are employed. In the first method, a direct hydrolysis of the sample with 2 N H_2SO_4 in a boiling water bath is used, as outlined by Steele (3), with subsequent adsorption on Lloyd's reagent and elution with 0.4 N NaOH. The second, a water extract, is obtained, which is then hydrolyzed by enzymatic treatment and finally on a boiling water bath with hydrochloric acid. These extracts, for which data are tabulated in Table I, are obtained as follows, with wheat bran as an example. A clean wheat bran,

as free of screenings and of germ and endosperm as commercial milling permits, was ground in a Wiley mill (laboratory model) through a 30 mesh screen. 5 gm. of this material are suspended in 75 ml. of 2.0 N H_2SO_4 in a 100 ml. volumetric flask and hydrolyzed in boiling water for

TABLE I

Comparative Determinations of Nicotinic Acid

Materials	Microbiological	Collaborative	Bina, Thomas, and Brown
	γ *per gm.*	γ *per gm.*	γ *per gm.*
Barley, No. 2 Valley	65.0	43.4	38.0
" malt, No. 2 Valley	71.0	46.0	47.0
Wheat, No. 3 HRW	53.0	38.0	40.0
Oats, No. 1 white	7.2	6.8	6.9
Rye	8.7	7.3	7.4
Corn, yellow	15.7	12.5	11.5
Millet, E. fortune	25.2	17.3	15.8
Milo maize	22.3	17.2	17.5
Sargo, Atlas	25.0	22.5	20.8
Flax, Viking	32.5	24.0	23.3
" Bison	23.6	19.2	21.5
Rice, brown	54.9	43.5	47.4
" rough	59.0	40.0	43.5
" milled	11.8	11.4	9.6
Peanuts, runner	125.0	111.6	123.8
Cow-pea, Canada	20.0	17.7	19.6
Soy bean, Illinois	16.0	10.0	10.7
Lima "	11.3	19.5	20.3
Navy "	10.6	17.3	17.6
Black eyed peas	17.2	16.5	23.4
Lentils	22.7	21.8	22.7
Sesame seed	42.5	34.5	43.3
Poppy "	8.2	9.8	10.7
Caraway-seed	21.2	21.1	21.3
Watermelon seed	15.1	13.0	13.7
Wheat bran	214.8	155.1	140.4
" germ	54.4	42.6	57.6
Bread, enriched	41.7	35.2	36.6
Powdered skim milk	10.0	14.0	12.0
Yeast, dried, brewers'	453.0	450.0	457.0
Distillers solubles, dry	52.5	46.0	53.0
Penicillin mold. dry	142.5	110.0	180.0

1 hour, with occasional mixing. The flask is cooled to room temperature and made to volume with distilled water. The contents of the flask are well mixed and a 25 ml. aliquot is transferred to a 50 ml. centrifuge tube and the pH adjusted to 0.5 to 1.0 with 40 per cent caustic solution,

with methyl violet on a spot plate as an outside indicator. 1 drop of indicator and 1 drop of the sample are mixed on the spot plate and the color compared with that produced when 1 drop of indicator is mixed with 1 drop of 0.2 N H_2SO_4. 1 gm. of Lloyd's reagent is then added and the contents of the tube are mixed well for at least 1 minute. The tube is then centrifuged clear and the supernatant liquid discarded. The Lloyd's reagent is washed twice by suspending it in 10 ml. of 0.2 N H_2SO_4, centrifuging, and discarding the supernatant liquid. After the final washing, the tube is inverted and allowed to drain well. 25 ml. of 0.4 N NaOH are then added and the contents mixed by stirring and shaking. The tubes are centrifuged and the supernatant liquid poured into other centrifuge tubes containing 1.6 gm. of powdered lead nitrate, and mixed well by swirling. 1 drop of phenolphthalein is added, the solution being decolorized if sufficient $Pb(NO_3)_2$ has been used. Solid K_3PO_4 is now added until the solution is pink. The pH is adjusted to between 5.0 and 6.0 with 10 per cent H_3PO_4 and K_3PO_4 with nitrazine paper as an outside indicator. The tubes are centrifuged and the supernatant liquid decanted carefully for color development and microbiological assay.

Three 5 ml. aliquots of each solution are used for color development in the chemical procedure, marked a, b, and c. 12 ml. of water are added to a, and 1 ml. of water to b. 1 ml. of standard niacin solution containing 10 γ of niacin is added to c. 1 ml. of CNBr reagent is then added to b and c, and after 15 minutes 10 ml. of the metol reagent are added. The samples are kept in the dark for 1 hour, after which the color is measured in a Pfaltz and Bauer photoelectric spectrophotometer at a wave-length of about 420 mμ with a combination blue and yellow filter. Metol is used to produce the color complex.

The second method is essentially the method of Bina, Thomas, and Brown (4), in which a water extract is prepared by suspending 10 gm. of ground sample in 125 ml. of distilled water and heating at 15 pounds pressure in an autoclave for 30 minutes, then cooling to 50°, and adding 0.4 gm. of taka-diastase. The sample is incubated for 2 hours and again autoclaved for 30 minutes at 15 pounds pressure, after which it is centrifuged and the residue washed twice with 15 ml. portions of hot water. The combined extract and washings are then hydrolyzed by treatment with 5 ml. of concentrated HCl and held in a boiling water bath for 30 minutes. After centrifuging, the acidity is adjusted to a pH of 6.4 to 6.8 and is ready for assay according to the procedure described by Bina, Thomas, and Brown, with p-aminoacetophenone as the color reagent. The results are tabulated in Table I, giving the values obtained for a number of products by these different methods.

Influence of Hydrogen Peroxide Treatment on Nicotinic Acid Values—

Aliquot portions of the samples of wheat bran and malt extracts, values for which are recorded in Table I, were used for treatment with hydrogen peroxide. 50 ml. portions of each of these extracts that had been prepared up to the final stage, including adsorption and elution with Lloyd's reagent, were treated with 5 ml. of 40 per cent NaOH and 5 ml. of 30 per cent hydrogen peroxide at 70° for 2 hours, then evaporated to dryness on the steam bath. The residues were again dissolved in distilled water, the pH adjusted to 6, then diluted to the original volume for nicotinic acid determinations.

500 γ of c.p. niacin were dissolved in 50 ml. of distilled water and treated with NaOH and hydrogen peroxide in exactly the same manner as described above, and the nicotinic acid determined along with the bran and malt samples by the microbiological procedure. The values obtained on these samples are recorded in Table II, in comparison with the values obtained on the untreated aliquot recorded in Table I.

TABLE II

Nicotinic Acid Values Obtained by Microbiological Procedure

Materials	Original extract	Oxidized extract
	γ per gm.	γ per gm.
Wheat bran............................	214.8	149.2
Barley malt...........................	71.0	52.5
Nicotinic acid, c.p...................	500.0	506.0

Effect of Infra-Red Radiation on Nicotinic Acid and Nicotinic Acid in Presence of Hydrogen Peroxide—Evaporation by means of infra-red radiation has become an effective method in the evaporation of liquids and the removal of moisture from difficult drying products. Experiments were made to determine whether infra-red radiation was applicable for use in the concentration of extracts containing nicotinic acid, as this procedure is faster and preferred to evaporation on a steam bath, provided it does not affect the product.

Two samples of c.p. nicotinic acid (500 γ) were dissolved in 50 ml. of distilled water, made alkaline with NaOH, and evaporated, Sample A on the steam bath and Sample B under a laboratory infra-red lamp. Each sample was dissolved in distilled water, neutralized, made to volume, and analyzed by the microbiological and chemical procedures. Complete recovery of the nicotinic acid was obtained by all three methods.

Two other samples of c.p. nicotinic acid were dissolved in 50 ml. of distilled water, 5 ml. of NaOH and 5 ml. of hydrogen peroxide added, and the solutions evaporated to dryness on the steam bath and under the infra-

red lamp as in the previous experiments. Analyses made on these solutions revealed that Sample A, which was treated with hydrogen peroxide and evaporated on the steam bath, was unaffected and gave complete recovery by each of the methods, while Sample B, evaporated under the infra-red lamp, showed complete destruction as determined by the chemical methods and 90 per cent by the microbiological procedure. These results are tabulated in Table III.

50 ml. aliquots of the wheat bran and barley malt extracts were treated with hydrogen peroxide, NaOH, and evaporated to dryness on the steam bath, and also by the infra-red lamp, exactly as previously described for

TABLE III

Results of Nicotinic Acid Analyses

Experiment No.	Nicotinic acid, c.p., 500 γ	Found	Per cent destroyed
		γ	
1	Evaporated on steam bath	500	None
2	" with infra-red lamp	500	"
3	Treated with H_2O_2 and steam bath	500	"
4	" " " " infra-red lamp	40	92

TABLE IV

Results of Analyses of Wheat Bran and Barley Malt Extracts

	Original	H_2O_2, steam bath	H_2O_2, infra-red
	γ *per gm.*	γ *per gm.*	γ *per gm.*
Wheat bran...............................	214.8	149.2	44.3
Barley malt...............................	71.0	52.5	12.1
Nicotinic acid, c.p., 500 γ.................	487.0	495.0	50.0

the treatment of samples recorded in Table II and paralleled with c.p. nicotinic acid similarly treated. The results of these analyses are recorded in Table IV, as determined microbiologically.

DISCUSSION

The values for nicotinic acid obtained on the different products listed in Table I represent the nicotinic acid content of these products as regularly determined by the microbiological and chemical methods. A comparison of these results shows that a majority of the cereal products give higher values by the microbiological method than by the chemical procedures. This is especially true with wheat and malt products, in which appreciably higher values are obtained on the same extracts by the microbiological procedure than by the chemical method. This is not the case,

however, with oats, rye, wheat germ, and yeast, in which approximately the same values are obtained by all methods. On the other hand, lima and navy beans give considerably higher values by the chemical than by the microbiological method. The values for sesame, poppy, caraway, and watermelon seeds are in good agreement by all the methods. The same is true of cow-peas, black eyed peas, and lentils, in which almost identical values are obtained. Penicillin mold gave different values by each method used, probably due in part to the fact that the extract obtained could not be completely decolorized for the determinations which may have supplied some color that interfered in the chemical procedures, increasing the values.

Oxidation of wheat bran and barley malt extracts with hydrogen peroxide materially lowers the nicotinic acid values obtained before the oxidation, as shown in Table IV. The nicotinic acid values on these oxidized extracts by the microbiological method are in close agreement with the values obtained on the unoxidized extracts by the chemical methods. It is advantageous to make the oxidation treatment with hydrogen peroxide on the nicotinic acid fraction after adsorption on Lloyd's reagent and separation from the hydrolysate. This separates the nicotinic acid fraction from a large amount of oxidizable material in the hydrolysate that also is oxidized when the reaction is carried out without this separation. This separation gives a more effective procedure in limiting the action of the hydrogen peroxide to the nicotinic acid fraction and eliminates loss of reagent used up by the other materials. Similar losses in nicotinic acid values are shown without this separation but require more reagent and time.

The use of the infra-red radiator to evaporate the extracts produced no deleterious effects on the nicotinic acid except in the presence of hydrogen peroxide. A temperature of 115° was maintained.

The hydrogen peroxide treatment produces no destruction of nicotinic acid when solutions are evaporated to dryness on the steam bath.

Nicotinic acid, c.p., treated with hydrogen peroxide and infra-red radiation, as described in the experiments, undergoes practically complete destruction, as shown in analyses by the microbiological procedure. Similar treatment, with cereal extracts, gave values far below the values obtained by the chemical methods or on the same oxidized extracts without the infra-red treatment, indicating destruction of nicotinic acid as well as interfering substances in the extracts, by the combination of these treatments.

The fact that nicotinic acid values vary appreciably with the method of hydrolysis employed in both the chemical and microbiological methods and that drastic treatments with acid or alkali are required to produce maximum niacin values for the products casts doubt on the availability

of the niacin to the human organism found by these methods on natural products. Water extracts and enzymatic treatment of natural products liberate only a portion of the total material that is liberated by the drastic hydrolytic methods usually employed for these analyses and indicate that a large percentage of the niacin material is not made available by the digestive process when these products are consumed as food. The fact that Goldberger and Wheeler (6) found wheat germ much better than whole wheat in their work with pellagra patients, even though analyses place them approximately equal in niacin values, points to the fact that either the niacin values by chemical and microbiological methods are incorrect or the material contained in whole wheat, bran, etc., is not as available as the niacin fraction contained in the wheat germ.

While animal methods are exceedingly difficult and at present limited to dogs, it would appear that a comprehensive bioassay of food materials for available niacin values would be justified. The large food requirements of the dog, the number of animals, and the length of time required would impose a considerable task, but the results obtained should clear up a number of points that appear to offer no other solution.

SUMMARY

Nicotinic acid determinations were made on a number of products by three different microbiological and chemical procedures. With some of the products good agreement was obtained by all of the methods, while with other products wide variations resulted. These variations depend as much on the product analyzed as upon the procedure used and point to the fact that the different methods are not specific for the same material.

Oxidation materially lowers the apparent nicotinic acid values of extracts of wheat and malt products but solutions of pure nicotinic acid are not affected by the same treatment. Infra-red radiation of nicotinic acid solutions in the presence of oxidizing reagents produced destruction of the nicotinic acid.

The drastic treatment required for the liberation of nicotinic acid from natural products is such that doubt is cast on the availability of large proportions of these values in human nutrition.

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PEA STARCH, A STARCH OF HIGH AMYLOSE CONTENT

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Two general classes of starch have been recognized up to the present time, the common starches, containing both amylose and amylopectin molecules, and the waxy or glutinous cereal starches, which are comprised almost entirely of amylopectin. The first class can, in turn, be subdivided into two broad groups, one composed of the so called root starches, such as tapioca, potato, and sweet potato starch, which contain about 17 to 22 per cent amylose, and the other comprising the cereal starches which contain approximately 25 to 29 per cent amylose.

The differences in physical properties exhibited by pastes, sols, and gels of starches of these two groups result, in part, from the difference in the ratio of amylose to amylopectin molecules; *i.e.*, of linear to non-linear molecules. Other important factors influence the differences in physical characteristics of starch-water systems of the two groups; these include the average chain length of the amylose, the amount of phosphoric acid directly bound to the amylopectin, and the degree of association of amylose and amylopectin.

One starch in a possible third group containing slightly over 30 per cent amylose has been described (1). This was obtained from Easter lily bulbs. Its paste characteristics are more like those of corn-starch than of the root starches and it may belong with the cereal starch group.

Mr. J. P. Nielsen of the Western Regional Research Laboratory noted, during a study of the colors given by a number of starches with iodine, that a peculiar coloration was obtained in the case of starch from garden type, wrinkled seeded peas (2). On the basis of this color, he believed the amylose content of wrinkled seeded pea starch to be exceptionally high, and estimated it to be about 75 per cent. Mr. Nielsen called this conclusion to the attention of the authors. Apparently the only other experimental work on pea starch was that carried out by Reichert (3) who reported no unusual characteristics. Because a starch having such a high content of amylose would be unique and might have unusual physical properties, a thorough study of it, as suggested by Mr. Nielsen, appeared desirable. Starch was therefore prepared from wrinkled seeded peas, and

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its amylose content determined by as exact methods as are at present at our disposal. Observations were made also on granule characteristics, such as microscopic appearance, gelatinization, and x-ray pattern, and paste properties, including viscosity and light transmittancy.

<center>EXPERIMENTAL</center>

Starch prepared from wrinkled seeded peas of the Alderman, Perfection, and Stratagem varieties was studied. Complete data were not collected on starch from all three varieties. Alderman variety, however, which was included in all studies, may be regarded as representative of the class. Polarimetric starch determination indicated that both the Alderman and Stratagem peas contained about 30 per cent starch on a dry basis.

For separation of the starch, whole peas were steeped for 24 hours at 40° or 49° in distilled water to which a few ml. of toluene were added to inhibit the growth of microorganisms. The peas were then processed by the method used by Cox, MacMasters, and Hilbert (4) for the preparation of starch from corn. Starch recovery was poor, averaging from 5 to 10 per cent of the total air-dry weight of the peas, or about 18 to 30 per cent of the total starch present. The starch recovered appeared representative with respect to microscopic appearance and granule size.

Moisture, nitrogen, phosphorus, and ash content of the starch and starch content of the peas were determined as described by MacMasters and Hilbert (5). Fatty acids were determined by the method used by Whistler and Hilbert (6).

Transmittancy curves of the starch during pasting were obtained by Morgan's (7) method with slight modifications made in the apparatus by S. A. Karjala (unpublished).

The procedure used to determine the gelatinization temperature of the starch granules was essentially that described by Reichert (3). Gelatinization temperature is defined as that temperature at which birefringence has been lost by all the granules.

Viscosity was determined in a MacMichael viscometer, as described by MacMasters, Eck, and Hilbert.[1]

The method described by Bates, French, and Rundle (8) and modified by Wilson, Schoch, and Hudson (9) was used for determining the iodine-sorptive capacity of starch and amylose. Before the determination was made, each sample was extracted for 48 hours with 85 per cent methanol in a Soxhlet extractor to remove fatty material. After this extraction, the starch contained about 0.05 per cent fatty material. Iodine-sorptive

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[1] MacMasters, M. M., Eck, J. W., and Hilbert, G. E., in preparation.

capacity is reported as mg. of iodine sorbed per gm. of starch or starch fraction.

Fractionation of pea starch was effected by a modification of Schoch's (10) method. Prior to fractionation, the starch was largely defatted by Soxhlet extraction for 48 hours with 85 per cent methanol, and was then pregelatinized by treatment with liquid ammonia and precipitation with alcohol, a method which is to be described elsewhere (J. E. Hodge, unpublished data). The gelatinized starch, dried out of alcohol, was suspended in water saturated with butanol at 90°. After 1 hour the system was allowed to cool slowly to room temperature. The crude amylose complex which separated was purified by immediate redispersion, without drying, by heating at 15 pounds gage pressure for 30 minutes in water saturated with butanol. The hot system was centrifuged to remove the appreciable quantity (about 5 per cent of the original starch) of ungelatinized granules still present, and again cooled slowly to permit formation of the purified amylose-butanol complex. The amylose was liberated from the complex in the manner described by Schoch (10).

The method of Schoch and Jensen (11) was used to determine the alkali lability value of the amylose.

Results

Physical Characteristics of Granules—Starch granules from the cotyledon of garden type, wrinkled seeded peas are, in general, approximately globular in form and rosette-shaped (Fig. 1), and, unlike most starches, do not exhibit a cross when viewed between crossed Nicol prisms. Rather, they show small wedge-shaped bright sectors, with the apex of each pointed toward the center of the granule (Fig. 2). Birefringence of the globular granule is, however, relatively weak. In starch from the Alderman variety, an occasional ellipsoidal granule was observed which exhibited a distinct cross. One of these which is strongly laminated is shown in Figs. 1 and 2 at the right of the field. None of these ellipsoidal granules were observed in the starch from the Perfection and Stratagem varieties of peas.

Each of the common globular granules has a number of cracks or cleavage planes radiating from the center. Pea starch from several garden type, wrinkled seeded varieties was studied by Reichert (3) who considered the deeply fissured granules to be "probably simple." Cleavage of the granule along the planes or fissures into several small fragments, usually four to six in number, is not uncommon. When viewed between crossed Nicol prisms, however, each fragment shows a cross. The whole appearance, as well as the behavior of the fragments upon heating in water, indicates that each globular granule is a compound granule, composed of several simple wedge-shaped granules which are the fragments formed by cleavage.

In Fig. 1, at the lower center, a compound granule is shown which is just beginning to break up into simple granules. Several simple granules and some intact compound granules are also shown in Figs. 1 and 2. Although generally only simple granules are found in the common commercial starches, it is well known that starch from some plants consists almost entirely of compound granules (3).

The compound granules of wrinkled seeded pea starch average from about 20 to 40 μ in diameter. The simple granules which comprise them are irregular in shape and hence difficult to measure, but are, roughly, about
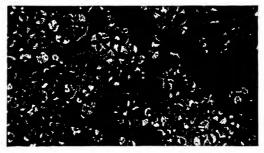
Fɪɢ. 1. Ungelatinized starch granules from the Alderman variety of wrinkled seeded peas. × 300.

Fɪɢ. 2. Same field as Fig. 1, between crossed Nicol prisms. × 300

twice as large as the average small granules of commercial wheat starch. Although some of the compound granules break up during the separation of the starch from the other constituents of the cotyledon, many remain intact when heated in water to 120°, the simple granules swelling somewhat *in situ.*

The most striking characteristic observed for starch from wrinkled seeded, garden type peas was the high gelatinization temperature. Practically all other known starches are completely gelatinized upon being heated in water to 75° or 80°. These pea starches, however, were only

partially swollen (Fig. 3) and still showed some birefringence (Fig. 4) after heating in water at 95–97° for 20 minutes, and even after 1 hour at 120°. These results are contrary to the findings of Reichert (3) who reported the gelatinization temperature of starch from garden type peas to be 72.5–75°. Starch from the Thomas Laxton variety of garden type peas, separated in Reichert's laboratory and presumably the sample which he studied, was found by the present authors to be incompletely gelatinized at 95°, similar to the samples freshly prepared in this Laboratory.

Fig. 3. Perfection variety pea starch granules heated in water at 95–97° for 20 minutes. × 300.

Fig. 4. Same field as Fig. 3, between crossed Nicol prisms. × 300

Starch from wrinkled seeded peas either in the gelatinized or ungelatinized state gives with iodine a clear blue color, with no trace of a violet tinge such as is shown by the cereal starches. Root starches also, however, give a clear blue coloration with iodine solution. These variations in the coloration with iodine are apparently superficial and unrelated to amylose content.

Wrinkled seeded pea starch is the first among the large number of seed starches studied in this Laboratory that has been found to give the B x-ray pattern, typical of potato starch. In the pea starch x-ray pattern, the line number 5 (12) corresponding to the interplaner spacing of 4.47 A

is slightly stronger than that of the typical B pattern; otherwise the two are identical.

Non-Carbohydrate Constituents of Granules—Starch from wrinkled seeded peas has, in general, an amount of non-carbohydrate constituents intermediate between that in corn-starch and that in potato starch. Comparative data on samples of the three starches similarly prepared in the laboratory are given in Table I. The high nitrogen content of the pea starch is attributable to the great difficulty encountered in separating the starch from the protein which occurs with it in the cotyledons. Large pieces of this proteinaceous material were microscopically visible in the finished starch samples. The characteristics of the starch suspensions are not attributable to contamination of the sample with protein, however, for equally high protein content has only a relatively minor effect upon the

TABLE I

Non-Carbohydrate Constituents of Starch

All values are on the dry basis.

Constituent	Alderman pea starch	Corn-starch	Potato starch
	per cent	*per cent*	*per cent*
Nitrogen........................	0.60	0.05	0.01
Phosphorus......................	0.043	0.020	0.085
Ash............................	0.13	0.06	0.30
Fatty acid, by weight.............	0.31	0.70	0.07
" " " titration..........	0.13	0.45	

properties of starch pastes, and no effect upon the swelling characteristics of starch granules.

Physical Characteristics of "Paste"—Because of the limited degree of granule swelling, "pastes" of wrinkled seeded pea starch, prepared by heating 5 per cent starch in water for 1 hour at 95°, have the characteristics of suspensions rather than of pastes. The system is opaque and not very gelatinous. The granules rapidly settle when the "paste" is allowed to stand without stirring. A 5 per cent corn-starch suspension heated to 80° has the same general appearance. When the pea starch-water system is allowed to cool to room temperature, a two-layered gel is formed, composed of a very stiff layer below one which is only moderately stiff. Layering is the result of the settling of the granules.

The low swelling capacity of wrinkled seeded pea starch is reflected in the viscosity of its "pastes." Even upon prolonged heating in water, the viscosity at 90° of wrinkled seeded pea starch-water systems is very low, being much less than that of the common and glutinous starches. The

viscosity is increased but little when high stirring rates are used to break the compound granules into simple granules in an endeavor to permit complete swelling of the latter. Under these high velocity stirring conditions there was no apparent dispersion or breakage of the simple granules. Characteristic viscosity data are given in Table II.

Light transmittancy curves obtained during the heating of 0.1 per cent pea starch in water are relatively flat and without inflection (Fig. 5). In this, pea starch is unlike corn, potato, and other starches, the light transmittancy curves of which have a sharp inflection point in the neighborhood of the gelatinization temperature. The much greater opacity of suspensions of starch from wrinkled seeded peas at elevated temperatures is due to the limited swelling of the granules.

Amylose Content of Starch—The amylose content of starch from wrinkled seeded peas is much higher than has previously been reported for any

TABLE II

Viscosity of Starch from Alderman Peas

5 per cent starch suspension heated 60 minutes at 95°.

Rate of stirring during heating	Apparent viscosity at 90°
revolutions per min.	*centipoises*
120	2.7
1000	2.7
1800	3.6

starch. This conclusion is reached on the basis both of iodine-sorptive capacity of the starch (8) and of the results of fractionation with butanol (10). Although the iodine-sorptive capacity of the starch depends to some extent upon the variety of the peas from which it is obtained, all of the samples studied sorbed over 100 mg. of iodine per gm. of starch. If the approximate sorptive capacity of 200 mg. of iodine per gm. of amylose is taken as a standard for pure amylose, the amylose content of the pea starches studied is calculated to be between 60 and 70 per cent. Data and the calculated amylose content are given in Table III.

Upon fractionation of Alderman pea starch, a 60 per cent yield of amylose was obtained. This fraction had an iodine-sorptive capacity of 187 mg. per gm., indicating about 93 per cent amylose content. This is about the purity obtained for corn amylose under the same conditions of fractionation. The alkali lability value for the pea amylose fraction was 15.8, thus indicating that its molecular size approximates that of corn amylose and is appreciably less than that of potato amylose.
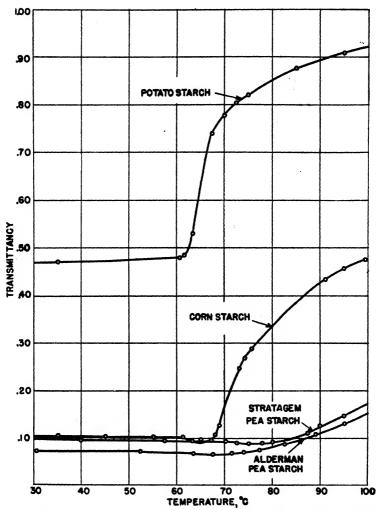
Fig. 5. Light transmittancy curves of corn, potato, and wrinkled seeded pea starch in 0.1 per cent suspension.

TABLE III

Iodine-Sorptive Capacity and Amylose Content of Wrinkled Seeded Pea Starches

Source of starch	Iodine-sorptive capacity	Approximate amylose content*
	mg. per gm.	*per cent*
Alderman peas..................	130	65
Perfection "	138	69
Stratagem "	120	60

* Calculated on the assumption that pure pea amylose sorbs 200 mg. of I per gm.

DISCUSSION

The physical characteristics of wrinkled seeded pea starch are consistent with its high amylose content. Amylose, because of its high association power, is not readily dispersed to form colloidal solutions at temperatures below 100°. It is not surprising, therefore, that granules containing largely amylose are but little swollen under ordinary pasting conditions. In contrast is the behavior of glutinous, or waxy, cereal starches, the granules of which consist essentially of amylopectin, contain practically no amylose, and swell greatly at relatively low temperatures to form viscous, tacky pastes. There is no record in the literature of a starch that cannot be dispersed by heating in water at 120° for an hour. Since a large portion of pea starch is still incompletely gelatinized after this treatment, the amylose is presumed to be in an unusually highly associated state.

Starch from wrinkled seeded peas cannot be classified in any of the starch groups known up to the present time. It has approximately twice the maximum amylose content previously reported for any starch and possesses unusual swelling properties. A new starch group must therefore be recognized in which the granule and paste characteristics are markedly influenced by an exceptionally high amylose content.

At present, starch from the various varieties of wrinkled seeded peas is the only kind known to belong in this group. Starch from smooth seeded, field type peas is essentially like the common cereal and root starches in regard to its amylose content.

SUMMARY

Starch belonging to an entirely new class has been found to occur in three varieties of garden type, wrinkled seeded peas. The granules do not gelatinize completely even after prolonged heating in a boiling water bath. As a consequence, the pea starch-water systems heated to 97° resemble suspensions rather than pastes. A paste is not formed even after heating the system to 120° for 1 hour. Iodine-sorptive capacity and fractionation of the starch show the amylose content to be 60 to 70 per cent, depending upon the variety of peas. No starch has hitherto been reported with so high an amylose content, or such unusual physical characteristics.

The authors wish to express their appreciation for the suggestion of Mr. J. P. Nielsen that garden type, wrinkled seeded pea starch might contain an unusually high percentage of amylose.

Grateful acknowledgments are made of the collection of pea samples by the Commodity Development Division, of the x-ray data obtained by Dr. N. C. Schieltz, of the analytical determinations made by members of the Analytical and Physical Chemical Division, and of the laboratory aid

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THE PRESENCE OF CREATINE IN THE TESTES OF VARIOUS INVERTEBRATES. THE PREPARATION OF CREATINE PHOSPHORIC ACID FROM FISH TESTES*

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According to Kutscher and Ackermann (1–3), there is a very marked difference between vertebrate and invertebrate animals in that the latter do not contain creatine, but, in its place, arginine. After the discovery of creatine phosphoric acid by Fiske and Subbarow (4, 5) and by Eggleton and Eggleton (6), it became of interest to see whether arginine in invertebrate muscle was similarly present in combination with phosphoric acid. This was shown to be the case for the muscle of crawfish by Meyerhof and Lohmann (7), and for those of *Octopus* by Lohmann (8). Indirect evidence, chiefly that of the rate of hydrolysis of organic phosphoric acid and the effect thereon of the presence of molybdate ions, has indicated the presence of arginine phosphoric acid in the muscles of other invertebrate forms, including other mollusks and arthropods, platyhelminths, nemertines, annelids, podaxonia, and echinoderms (9–13).

It seemed to be of interest to determine whether the forms that are generally believed to be intermediate between the achordates and the true vertebrates contained creatine. Accordingly, in 1929, analyses were made of the muscles of an ascidian, *Boltenia*, which is found near the Mount Desert Island Biological Laboratory. The teased muscle fibrils of the basket contained a substance which, after heating with acid, reacted with alkaline picrate solution with the production of an orange color. The intensity of this reaction indicated an apparent concentration of from 25 to 42 mg. of creatine per 100 gm. of tissue (Table I). Neither the facilities nor the material available permitted further attempt at identification.

The conspicuous mixed gonads of *Boltenia* were found to contain from 36 to 92 mg. of apparent creatine per 100 gm. of tissue. Without heating, the same acid extracts seemed to contain 12 to 25 mg. of apparent creatine per 100 gm. of tissue. About 150 gm. of gonads were collected, preserved by heating in water, and brought to New York for an attempt at isolation of the creatine as potassium creatinine picrate. This was unsuccessful. In the course of this attempt, 0.190 gm. of betaine picrate was obtained, indicating a concentration of at least 44 mg. of betaine per 100 gm. of tissue.

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* Some of the material contained in this paper was reported to the Fourteenth International Physiological Congress, Rome, 1932.

The only other invertebrate containing any appreciable quantity of testes or sperm that was available at Mount Desert Island at that time was *Echiurus.* The sperm-laden nephridia, when extracted with cold 0.1 N HCl, yielded only the equivalent of 0.9 mg. of creatinine per 100 gm. After

TABLE I

Determination of "Apparent Creatine" in Various Fresh Tissues

Species	Tissue	No. of analysis	Concentration	
			Range	Average
			mg. per 100 gm.	*mg. per 100 gm.*
Bollenia..................	Fibrils of basket	4	25–42	31
	Mixed gonad	9	36–92	59
Echiurus.................	Sperm-laden nephridia	1	144	144
	Muscle	4	23–40	31
	" not heated	3	1–18	10
Loligo...................	Ovaries	2	31–52	42
	Testes	9	62–97	82
	Vas deferens	5	72–229	163
	Spermatophore organ	6	44–208	98
Ostrea...................	Ovaries	5	31–41	35
	Testes	5	57–67	63
Whelk...................	" and vas deferens	3	<27–<38	
Callinectes...............	Testes	4	28–39	33
	Vas deferens	4	42–55	47
Arbacia*.................	Ovaries	4	19–52	39
	Testes	<5	154–214	189
Asterias*................	Ovaries	15	11–21	17
	Testes, immature	5	27–94	53
	" mature	13	122–207	169
Strongylocentrotus.........	Ovaries	36	30–64	44
	Testes, Nov.	7	108–177	152
	" Dec.	21	98–222	163
	" Apr.	5	242–320	270
Ascaris†.................	Testes	2	27–35	31

* These data were obtained by Dr. Milton Levy. The labile organic specimens of starfish testes varied from 12.3 to 25.3 mg., average 20.0 mg., per 100 gm. of tissue. These values correspond to 52 to 107, average 85 mg., of creatine.

† These data were obtained by Dr. A. Lo Presti.

heating with acid, the amount was greatly increased, indicating the presence of 144 mg. of creatine per 100 gm. No additional specimens of *Echiurus* could be collected at that time, but Dr. D. M. Whitaker was kind enough to obtain the sperm from several specimens of *Urechis* collected at Morrow Bay, California. This was preserved in alcohol and sent to New

York. From this material, creatine was isolated as potassium creatinine picrate.

It seemed possible that the presence of creatine in other invertebrates had been overlooked by previous investigators, who either confined their attention to the muscles or used entire animals. If these were not sexually mature at the time, any creatine that sperm might contain would not have been detected.

It was decided to investigate such animals as might be available, when sexually mature, in quantity in the markets of New York. It was found that while small amounts of chromogenic material were present in the heated acid extracts of the testes of certain mollusks (squid, whelk, oyster) or of arthropods (crab), the testes of the sea urchin (*Strongylocentrotus*) contained very considerable quantities. This was first observed early in the season with rather immature animals, in which the apparent creatine content was about 100 mg. per 100 gm. As development proceeded, the concentration of creatine steadily increased to reach, in some cases, as much as 320 mg. per 100 gm. The ovaries, whether mature or not, had an apparent creatine content of only 30 to 50 mg. per 100 gm.

Without heating, the dilute acid extracts of a large quantity of testes contained only about 10 mg. of "apparent creatinine" per 100 gm. of tissue. After heating, this was increased to about 90 mg. From these acid extracts, creatine was isolated either as such, or as creatinine potassium picrate in amounts that corresponded quite well with those estimated by Jaffe's reaction.

If comparison were made with the figures obtained by heating the entire tissue, the agreement was not so good. Either some creatine was not extracted in the cold, or some other substance yielded a chromogenic material on heating with acid. In one experiment, the dilute acid *extract* contained 84 mg. of apparent creatine per 100 gm. of tissue. The residue, after thorough extraction with cold dilute acid, alcohol, and ether, was also heated with 0.1 N HCl. An apparent creatine content of 67 mg. per 100 gm. of fresh tissue was found.

The testes of squid (*Loligo*) were submitted to a similar examination. Whereas the entire testes seemed to contain from 70 to 116 mg. of creatine per 100 gm., the acid extract contained only from 28 to 32 mg. and, after correcting for the color developed without previous heating, only from 13 to 17 mg. It is believed that these tissues contain chromogenic substances other than creatine or creatinine and that values of 50 mg. or less of apparent creatine in any of our material are probably due to the presence of such other chromogenic materials.

The investigation then developed in two directions. The question arose, is the creatine present in sea urchin testes present as creatine phosphoric

acid? Dr. Milton Levy undertook to answer this question at Woods Hole. The sea urchins available yielded too little material, but starfish (*Asterias forbesi*) were more satisfactory. He found that the testes, but not the ovaries, yielded a large amount of chromogenic substance on heating with dilute acid, and that the testes contained a corresponding amount of labile phosphoric acid.

A number of attempts were made to prepare pure creatine phosphoric acid from the sea urchins available in New York or from vertebrate testes. Most of these were unsuccessful. However, after substitution of picric acid for trichloroacetic acid in extracting the tissues, it was a simple matter to prepare the calcium salt in pure form and quite satisfactory yield from the testes of the carp.

The other part of the investigation was concerned with the possible occurrence of creatine in other forms. These were collected at Roscoff, Concarneau, and Banyuls-sur-Mer in France, at Naples, at Morrow Bay, California, and at St. Andrews, New Brunswick. As is evident from Table II, the concentration in the testes or in the sperm-laden portions of some of the animals far exceeds that found in others. As explained above, it is believed that the lower values do not represent creatine at all. However, there can be little question but that values of 100 mg. or more of apparent creatine per 100 gm. of tissue are due largely to creatine. The substance has actually been isolated from three or four forms, representing two different phyla of invertebrates. It would appear, therefore, that creatine is a constituent of the sperm of annelids, Echiuridae, echinoderms, Tunicata, and Balanoglossidae, but is probably absent from that of mollusks and Crustacea, and presumably of other arthropods as well.

Since this work was begun, several papers on the subject have appeared. Reference has already been made to the isolation of arginine phosphoric acid from the muscles of *Astacus fluviatilis* and *Octopus vulgaris* (7, 8). In addition, Eggleton and Eggleton (14) showed that *Amphioxus* contains a phosphagen that resembles creatine phosphoric acid in its rate of hydrolysis and in the effect of molybdic acid upon this hydrolysis. Colorimetric estimations indicated a creatine content equal to that of the skeletal muscle of vertebrates. They were unable to find any evidence of the presence of such a phosphagen in the muscle of representative arthropods, mollusks, echinoderms, annelids, and coelenterates, nor did they find any colorimetric evidence of the existence of creatine in any of these.

Riesser and Hansen (15) came to a very different conclusion; *viz.*, that creatine phosphoric acid is of general occurrence among invertebrates. However, as Needham and associates (16) pointed out, the precipitation of inorganic phosphate by Riesser and Hansen was probably not complete and that what Riesser and Hansen determined was merely phosphate not precipitated by magnesia mixture.

While the rate of decomposition of creatine phosphoric acid is greatly accelerated by molybdic acid, that of arginine phosphoric acid is retarded. Using these facts as criteria, Needham, Needham, Baldwin, and Yudkin (10) undertook a comprehensive survey of invertebrates for the presence of these two phosphagens. The jaw muscles of *Strongylocentrotus* seem to contain both. *Although Walpole's diacetyl test for creatine was negative,* the results obtained by the colorimetric estimation of creatinine after heating in an autoclave yielded values that agreed with those calculated from the

TABLE II

Determination of "Apparent Creatine" in Various Tissues Preserved with Alcohol

Species	Tissue	Collected at	Crea-tine
			mg. per 100 gm.
Maia	Testes	Concarneu	30
Langoustine	"	"	36
"	Ovaries	"	46
Sipunculus nudus	Undifferentiated sex gland	"	25
Sabellaria alveolata	Females	"	39
	Males	"	66
Annelid, unidentified	Sperm-laden segments	"	385
Chaetopterus vareopedatus	Posterior "	Naples	108
Diopatra neopolitana	" "	"	237
Andovinia filigera	Whole	"	266
Balanoglossus	Proboscis and collar	"	312
Glossobalanus sarniensis	" " "	Concarneau	203
" "	Piece with ripe sperm	"	250
Urechis caupo*	Spermatozoa	Morrow Bay	688†
" " *	Nephridia with sperm	" "	707†
Microcosmus sulcatus	Mixed gonad	Banyuls-sur-Mer	98
Holothuria tubulosa	Testes	"	51‡
Cucumaria frondosa§	"	St. Andrews, N. B.	92‡

* Collected by Dr. D. M. Whitaker.
† Per 100 gm. of dried material previously extracted with alcohol and ether.
‡ In aqueous extract.
§ Collected by Dr. A. H. Leim.

rate of liberation of phosphoric acid. This is true only if one assumes the same degree of conversion (40 per cent) of creatine into creatinine in the extracts of muscle as occurred in the controls with solutions of creatine. From the description given by the authors ((10) p. 265), it would appear that they heated the filtrates from a precipitation with copper sulfate and calcium hydroxide without previous acidification. Under these conditions, it is not surprising that the conversion of creatine into creatinine was not quantitative, and it is also much more doubtful that the chromogenic material was creatinine.

Needham and associates found no creatine phosphoric acid in the muscles of ascidians. This agrees with the work of Flössner (17). He used the internal mantles of 7000 specimens, mostly *Ciona intestinalis*, with some *Phallusia mammilata*. This furnished enough material to yield 1.3 gm. of adenine picrate and 0.8 gm. of hypoxanthine silver; yet the extracts were negative to Weyl's and Jaffe's tests. It would appear, therefore, that the chromogenic material obtained by the present writer on heating the muscle fibrils of *Boltenia* with acid was not creatinine.

For *Balanoglossus*, Needham and associates reported from 0.039 to 0.077 mg. of labile phosphorus resembling creatine phosphoric acid per gm. of muscle in various parts of the body. A mixture of the extracts of these tissues, calculated from the labile phosphorus content to contain 0.68 mg. of creatine, yielded 1.10 and 0.90 mg. when analyzed by Walpole's diacetyl method and 0.82, 0.86, and 0.91 mg. when heated in an autoclave and analyzed with the Jaffe reaction, and a conversion factor of 60 per cent.

Our figure for the proboscis and collar region of *Balanoglossus* is 3.12 mg. of creatine per gm., or more than 12 times that calculated from the labile phosphorus reported for the same region by Needham, Needham, Baldwin, and Yudkin.

These workers found no evidence for the presence of creatine phosphoric acid in the muscles of any of the annelids examined. Our own results for *Sabellaria alveolata* are in accord with this. However, the high values for creatine found in the sperm-laden segments of a number of annelids were in all probability due to the actual presence of creatine. In the case of *Urechis caupo* this was confirmed by actual isolation of potassium creatinine picrate.

Using the rate of hydrolysis as indicator, Needham and associates (10) found only arginine phosphoric acid in the muscles of *Synapta inhaerens*. Adding to this the demonstration of arginine by means of arginase, Meyerhof (9) found only arginine phosphoric acid in the muscles of *Holothuria tubulosa*. In another species of the same order, *Cucumaria frondosa*, Verjbinskaya, Borsuk, and Kreps (13) found that the rates of hydrolysis indicated the presence of both creatine and arginine phosphoric acids.

In 1936, Baldwin and Needham (18) reinvestigated the nature of the phosphagens in echinoderms. Their criterion was the formation of such compounds by tissue extracts in the presence of added creatine or arginine, 3-phosphoglycerate, and adenylate or adenyl pyrophosphate.

With extracts of *Cucumaria frondosa*, they found phosphagen to be formed only when arginine was present. However, with extracts of *Paracentrotus lividus* and *Sphaerechinus granularis*, they found that phosphagens were formed from both bases.

The identity of the substance formed from arginine was confirmed by precipitation of the barium salt, with subsequent hydrolysis to yield a mate-

rial giving Sakaguchi's reaction and forming urea, when treated with arginase.

There was no such confirmation of the formation of creatine phosphoric acid. This was determined as the phosphorus not precipitated by barium acetate and "soda" at pH 8.5, yet reacting with the Fiske-Subbarow reagents within 30 minutes. According to a letter from Dr. Baldwin, "soda" was sodium hydroxide, not carbonate, and the protein trichloroacetate was not removed before adding the barium acetate and alkalinizing to phenolphthalein. After centrifuging, the supernatant fluid was treated with the Fiske-Subbarow reagents. Baldwin and Needham seem to have overlooked the fact that some protein trichloroacetate would be expected to dissolve and that the excess of barium acetate would reduce the acidity of the mixtures. Since the latter might lead to a reduction of molybdic acid by some tissue constituent, the evidence adduced for the presence of the specific enzymes cannot be regarded as satisfactory. Still less can it be held to support the view that creatine phosphoric acid is actually present in echinoid muscle. Urease is present in the soy bean and in the jack bean, but there is nothing to indicate that urea plays any part in the physiology of these beans.

. It is not the purpose of this paper to discuss the various suggestions that have been made regarding the genetic relations of the various phyla. However, the proved presence of creatine in Echinodermata and in Echiuridae and its probable presence in other Annulata point to a genetic relation of these phyla to the chordates, while the absence of creatine·from the testes of nematodes, arthropods, and mollusks seems to indicate that these phyla stand in no such relation to the chordates, but represent one or more entirely different lines of development.

I am indebted to the directors and staffs of the Mount Desert Island Biological Laboratory, of the Hopkins Marine Station, Pacific Grove, of the Biological Station, Saint Andrews, New Brunswick, of the Laboratoire de Zoologie maritime, Banyuls-sur-Mer, of the Laboratoire de Zoologie maritime, Roscoff, of the Laboratoire de Zoologie et de Physiologie maritimes, Concarneau, and of the Stazione zoologica di Napoli for their cooperation in securing specimens, and to Dr. Milton Levy and to Dr. A. Lo Presti for permission to publish their results.

EXPERIMENTAL

The method employed for the determination of creatine in fresh tissues was a slight modification of that of Ochoa and Valdecasas (19). Larger amounts of tissue were used, and, after the addition of picric acid, the mixtures were diluted to a definite volume. After filtration, aliquots were used for the determinations.

The materials collected in France and Italy were weighed and transferred to alcohol. Several months later the contents of the bottles, except the *Microcosmus* and *Holothuria* material, were transferred to beakers, 1 ml. of 2 N HCl added for each gm. of tissue, and the mixture heated on the water bath to drive off the alcohol. They were then diluted to a volume in ml. equal to 10 times the weight of the tissue in gm. After heating at 125° for 40 minutes, the mixture was treated with an excess of solid picric acid, diluted to a definite volume, and after thorough shaking and saturation filtered. An aliquot was taken for analysis.

Isolation of Creatine and Creatinine Potassium Picrate

(a) *From Testes of Strongylocentrotus*—The testes were dropped into 1500 ml. of alcohol as soon as they were removed from the animals. A total of 1246 gm. was used. After being stirred thoroughly, the material was set aside overnight. It was then filtered through muslin. The residue was extracted three times with alcohol and three times with ether. The alcohol extracts were added, and the mixture was allowed to stand. The precipitate was filtered out, taken up in water, acidified with HCl, and filtered. Analyses indicated the presence of 0.222 gm. of creatine. The remainder of the solution was treated with basic lead acetate, filtered, and the filtrate was freed of lead with H_2S and then evaporated at 20 mm. to small volume. On standing, crystals separated. These were recrystallized from water. The yield was 0.202 gm. Upon drying at 100° they lost 12 per cent water. Calculation for creatine hydrate, 12.06 per cent.

The material insoluble in alcohol and ether was air-dried and then extracted three times with water acidified to Congo red with hydrochloric acid. The combined extracts contained 794 mg. of creatine. They were evaporated at 20 mm. to small volume and the glycogen was precipitated with an equal volume of alcohol. After dilution and treatment with lead acetate and H_2S, the filtrate was again evaporated *in vacuo*. Upon standing, 0.267 gm. of crystals was obtained. These contained 12.2 per cent water. They were combined with those previously obtained. 1.49 mg. of the mixture yielded 1.28 mg. of creatinine; calculated, 1.285.

The filtrates from the creatine were made strongly acid with HCl and evaporated to dryness on the water bath. The residue was extracted with alcohol and this extract in turn was evaporated. This was continued until there was no residue insoluble in alcohol. The residue was then taken up in water and warmed with 0.32 gm. of K_2CO_3 and 3.6 gm. of picric acid. The crystals that separated on cooling were recrystallized. They weighed 2.4 gm., melted at 240–242°, and contained 75 per cent picric acid and 18.46 per cent creatinine. Calculated for potassium creatinine picrate, picric acid 75.2 per cent, and creatinine 18.55 per cent.

The total amount of creatine in the extracts was 1.016 gm. Of this, there were isolated as the hydrate 0.413 gm. and as potassium creatinine picrate 0.516 gm., a total of 0.929 gm.

(b) *From Urechis caupo*—The material consisted of two specimens collected by Dr. Whitaker. One consisted of sperm expressed from living animals, the other of sperm-laden nephridia obtained by dissection. The specimens had been placed in about 10 volumes of alcohol. Upon receipt, the alcohol was decanted and the insoluble material was twice extracted with ether. The combined alcohol and ether extracts were evaporated at a low temperature and lipids were precipitated by the method of Koch and Woods (20). The materials insoluble in alcohol and ether were dried, weighed, and powdered. They were then mixed with 30 or 40 volumes of water and acidified to Congo red with H_2SO_4. After 2 days in the ice chest, these mixtures were filtered and the filtrates were combined with the filtrates from the lipid precipitation. Aliquots were taken for a determination of the creatine. The remainder of the extracts, having an apparent creatine content of 22 mg. of creatine, was combined, treated with lead acetate, and then with H_2S. The filtrate was treated with HCl and evaporated to dryness. After repeated extraction with alcohol and evaporation until no material insoluble in alcohol remained, 10 mg. of K_2CO_3 and 70 mg. of picric acid were added. There were obtained 54.2 mg. of crystals melting at about 255°, and containing 75.2 per cent picric acid and 18.6 per cent creatinine. The crystals accounted for 53 per cent of the apparent creatinine content of the combined extracts.

(c) *From Microcosmus sulcatus*—145 gm. of the mixed gonads were preserved in alcohol. After 9 months, the alcohol was decanted and the residue was extracted with absolute alcohol and then with ether. The combined alcohol extracts were evaporated at low temperature. The ether extract was added and the mixture was extracted in a separatory funnel with several changes of water. The material insoluble in alcohol and ether was ground to a powder and extracted overnight with 300 ml. of water at 4°. This was added to the aqueous washings of the alcohol-ether extract. Analysis indicated the presence of 143 mg. of creatine. Attempts at its isolation as creatine zinc chloride or as potassium creatinine picrate failed. However, more than 70 per cent of the creatine could be accounted for in the chromogenic power of the picrates obtained.

(d) *From Holothuria tubulosa*—105 gm. of testes were preserved in alcohol and subsequently treated in the same manner as the *Urechis* sperm. The combined aqueous extracts contained 54 mg. of apparent creatine. The protein residue, on autoclaving, yielded 28 mg. The aqueous extracts yielded 0.1155 gm. of a potassium creatinine picrate, which melted at 255°, but contained only 73.4 per cent picric acid instead of the expected 75.2 per

cent, and only 17.2 per cent creatinine instead of the expected 18.55 per cent. The chromogenic value of this picrate accounted for 39 per cent of the total apparent creatine in the original extracts.

(e) *From Cucumaria frondosa*—29 gm. of testes were kindly collected by Dr. A. H. Leim in 1941 and placed in 285 ml. of 95 per cent alcohol. In 1945, they were treated in much the same manner as described under (a). Analysis of the acid aqueous extract indicated the presence of 27 mg. of creatine, or 92 mg. per 100 gm. of fresh tissue. In spite of the loss of about half of the material, there were isolated 36 mg. of a picrate decomposing at 245° and containing 75.8 per cent picric acid and 17 per cent creatinine.

TABLE III

Isolation and Identification of Calcium Salt of Creatine Phosphoric Acid from Testes of Carp

Per 100 gm. of tissue	Preparation 127	Preparation 135	Preparation 141
	mg.	*mg.*	*mg.*
Phosphorus, precipitated by $Ca(OH)_2$......	25.0	28.8	14.6
" labile in filtrate..............	25.2	22.9	30.0
Creatine, in filtrate.......................	100	145	155
Total tissue...........................	440 (gm.)		195 (gm.)
Yield.................................	115 (mg.)		131 (mg.)
	per cent		*per cent*
Calcium, calculated for $C_4H_8O_5N_3PCa \cdot 4H_2O$, 12.47%	12.9		12.46
Phosphorus, calculated for $C_4H_8O_5N_3PCa \cdot 4H_2O$, 9.66%	9.63		9.62
Creatine, calculated for $C_4H_8O_5N_3PCa \cdot 4H_2O$, 40.8%	40.5		40.3

Preparation of Calcium Salt of Creatine Phosphoric Acid from Testes of Carp—The fish were purchased in local markets and placed in tap water at room temperature. The receptacle was placed in the cold room (5°) and the water was aerated continuously. The next day the fish were opened and the testes were removed, weighed, and ground with solid picric acid. 4 ml. of a cold, saturated solution of picric acid were added for each gm. of testes. After being stirred for a few minutes, the mixture was filtered. The filtrate was treated with 0.1 volume of a 10 per cent solution of calcium chloride and enough sodium hydroxide solution to make it alkaline to phenolphthalein. The subsequent procedure was that of Fiske and Subbarow (5). One precipitation with 3 volumes of alcohol and three with 1 volume of alcohol yielded an almost colorless product that yielded satisfactory data upon analysis.

The precipitate produced by calcium chloride and sodium hydroxide was extracted with dilute nitric acid and saturated with picric acid. The insoluble material was filtered out and the phosphate in the filtrate was precipitated as ammonium phosphomolybdate. This was filtered out, washed, and dissolved in dilute ammonium hydroxide. Ammonium magnesium phosphate was then precipitated, removed by filtration, and weighed as such.

Labile phosphoric acid was estimated by acidifying a small portion of the alkaline filtrate, heating this to 70°, and then making it alkaline again. After standing overnight, the precipitate was centrifuged out, washed, and then used for a colorimetric determination of phosphorus (Table III).

SUMMARY

Creatine phosphoric acid was isolated as the calcium salt from the testes of the carp. The presence of creatine in the testes of *Strongylocentrotus, Holothuria tubulosa, Cucumaria frondosa,* and *Urechis caupo* was demonstrated by its isolation as such or as potassium creatinine picrate. In two ascidia (*Boltenia* and *Microcosmus*), in *Balanoglossus* and *Glossobalanus,* and in various annelids satisfactory isolation was not accomplished, but the amount of chromogenic material and some of its properties indicated very strongly that it actually was creatine. In the case of *Asterias forbesi*, the apparent creatine was accompanied by an equivalent amount of labile, organic phosphoric acid. In the testes of arthropods, mollusks, and of a nematode (*Ascaris*), the amount of chromogenic material was so low as to indicate that no creatine was present.

The phylogenetic significance of these observations is discussed briefly.

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THE EFFECT OF FAT ON THE UTILIZATION OF GALACTOSE BY THE ALBINO RAT*

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In 1938 Schantz, Elvehjem, and Hart (1) reported that rats, calves, and pigs which received liquid skim milk as their sole diet excreted a considerable amount of galactose in the urine. No such excretion occurred when whole milk was fed. In studies with the rat it was found that the addition of 3 to 4 per cent of fat to the skim milk completely prevented the urinary loss of galactose, and further investigation proved the fatty acid fraction of the fat to be responsible for this phenomenon (1, 2). The use of various synthetic triglycerides showed that the even numbered fatty acids of more than 10 carbon atoms were effective. Compounds such as choline, glycerol, etc., were ineffective in preventing the loss of galactose.

Zialcita and Mitchell (3) in 1945 concluded from studies made with synthetic rations containing lactose that fat as such had no influence on the utilization of galactose by the rat. The basal ration used consisted of lactose 48, fat 28, Salts 4 (4), and casein 20. Approximately equal urinary galactose losses were obtained when either butter fat or glucose comprised the "fat" portion of the diet. Somewhat lower excretions were found when the fat used was corn oil. The suggestion was made that corn oil might contain in the non-glyceride fraction some substance which influenced the metabolism of galactose.

In the present paper more detailed data are given concerning the influence of fat on galactose utilization when skim milk or synthetic rations containing lactose or galactose were fed to rats.

EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain weighing approximately 200 gm. were used in all of the experiments. Each animal was kept in

an individual wire mesh cage. 24 or 48 hour urine samples were collected under toluene with adequate precautions against contamination from either food or feces. A number of collections were taken on each regimen until constant galactose excretions were obtained. Food consumption records were kept. Sugar determinations were made by a slight modification of the Shaffer-Hartmann method (5), with the factor 1.22 to convert the values to galactose.

Studies with Milk Diets—Unpasteurized skim milk obtained daily from the University creamery was mineralized with 12 mg. of ferric pyrophosphate (Mallinckrodt N. F. VII), 0.6 mg. of $CuSO_4 \cdot 5H_2O$, and 0.6 mg. of $MnSO_4 \cdot 4H_2O$ per 100 cc. The liquid milk contained 4.65 per cent of lactose (equivalent to 2.45 per cent galactose). A commercial skim milk powder which contained 46.5 per cent lactose on a dry basis (equivalent to 24.5 per cent galactose) was mineralized so that each 10 gm. of powder contained the amounts of the minerals given above. Butter fat was prepared by decantation and filtration of the fat portion of melted unsalted sweet cream butter from the University creamery. To each 100 gm. of fat were added 2.24 mg. of α-tocopherol, 0.210 mg. of 2-methyl-1,4-naphthoquinone, 0.50 mg. of β-carotene, and 0.014 mg. of calciferol. The fat was incorporated into the liquid diets by means of a hand homogenizer. Each animal on the fat-free diets received 3 drops of corn oil per week which contained the fat-soluble vitamins at levels calculated to furnish the amount of each ingested by the fat-fed rats. Water was furnished *ad libitum* to all animals on the non-liquid rations.

Three rats were placed on each of the regimens shown in Table I, and the designated amount of each ration was offered to each animal daily. The galactose furnished per rat per day in Groups I to VI inclusive was 2.45 gm., provided all of the allotted ration was consumed.

The results of these experiments are shown in Table I. It is evident that the urinary excretion of galactose by rats fed either liquid skim milk or skim milk powder was markedly lowered when fat was incorporated into the ration. This decrease was not due to a reduced galactose intake by the animals receiving fat, for in Groups I and II five of the six rats each consumed 2.45 gm. of galactose daily. Of this amount those in Group I lost an average of 0.575 gm. in the urine ((ratio, excretion to ingestion) $\times$ 100 = 23.5), while only 0.080 gm. was excreted by those in Group II ((ratio, excretion to ingestion) $\times$ 100 = 3.27). The same fact is clearly evident in Groups VIII and IX. A comparison of Groups III to VI reveals no apparent effect of the large water intake on the loss of galactose.

To determine the effect of glucose on the utilization of galactose three rats were fed 100 cc. of skim milk until the average loss of sugar was con-

stant.   A supplement of 9 gm. of glucose was then given to each rat daily.
After constant losses were again obtained, the glucose addition was
stopped, and the animals were fed only the 100 cc. of skim milk.   The
data given in Table II show that the added glucose decreased the galactose
excretion to some extent.

Studies with Lactose Synthetic Diets—The effect of fat on the urinary
loss of galactose by rats fed lactose synthetic rations was studied with
the diets shown in Table III.   The ration designations "medium" and
"high plus liver" refer to the levels of B vitamins in each.   Twenty-four

TABLE I

*Influence of Fat on Galactose Excretion by Male Rats Fed Skim Milk or Skim Milk
Powder Rations* *

Galactose ingestion and excretion expressed in gm. per day.

Group No.	Ration per rat per day	Galactose ingested			Galactose excreted			$\frac{\text{Excretion}}{\text{Ingestion}} \times 100$		
		Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
I	100 cc. skim milk	2.45	2.45	2.45	0.545	0.377	0.804	22.2	15.4	32.8
II	100 " " " + 4 gm. butter fat	2.16	2.45	2.45	0.049	0.084	0.076	2.27	3.43	3.10
III	10.2 gm. skim milk powder	2.45	2.45	2.45	0.874	0.720	0.509	35.6	29.4	20.8
IV	10.2 " " " " + 4 gm. butter fat	1.96	1.81	1.85	0.191	0.100	0.100	9.74	5.53	5.40
V	10.2 gm. skim milk powder + H_2O to 100 cc.	2.45	2.45	2.45	0.425	0.538	0.626	17.3	22.0	25.5
VI	10.2 gm. skim milk powder + 4 gm. butter fat + H_2O to 100 cc.	2.45	1.52	1.65	0.147	0.035	0.035	6.00	2.30	2.12
VII	150 cc. skim milk	3.68	3.68	3.68	1.16	0.625	1.09	31.5	17.0	29.6
VIII	80 " " "	1.96	1.96	1.96	0.571	0.597	0.425	29.1	30.4	21.7
IX	80 " " " + 3.2 gm. butter fat	1.96	1.96	1.96	0.240	0.083	0.116	12.2	4.24	5.93

* Each figure represents the result from one rat.

male rats were divided into four groups; two groups received the medium
vitamin ration with butter fat and corn oil, respectively, and two groups,
the high vitamin ration with the two fats, respectively.   Both fats were
fortified with the fat-soluble vitamins at the levels given in the previous
section.

The animals had received their respective rations during a 6 weeks
growth period prior to being placed on this experiment.[1]   They were
maintained on the 48 per cent lactose level until constant urinary galac-

[1] For growth rates of these animals see Boutwell *et al.* (6).

TABLE II

Influence of Glucose on Galactose Excretion by Male Rats Fed 100 Cc. of Skim Milk per Day

Each figure represents the average of the same three animals.

Days on experiment	Glucose* per 100 cc. of milk	$\dfrac{\text{Galactose excretion}}{\text{Galactose ingestion}} \times 100$
	gm.	
20	0	39.3
27	0	37.3
37	Glucose supplements started	
56	9	17.7
61	9	20.6
76	9	18.5
77	Glucose supplements discontinued	
108	0	31.2

' Cerelose; commercial glucose monohydrate.

TABLE III

Composition of Lactose Synthetic Rations

Component	Vitamin level	
	Medium	High plus liver
	parts	*parts*
Carbohydrate*....................	48	48
Fat.............................	28	28
Casein†.........................	20	20
Salts 4‡.......................	4	4
Liver powder§.................	0	1
	mg. per 100 gm.	*mg. per 100 gm.*
Thiamine.......................	0.200	0.600
Riboflavin......................	0.300	0.900
Pyridoxine......................	0.300	0.900
Calcium pantothenate...........	1.500	6.000
Choline........................	100.00	200.00
Nicotinic acid..................		1.000
Inositol........................		100.00
p-Aminobenzoic acid...........		30.00

* α-Lactose (monohydrate) U. S. P. Merck, and *d*-glucose (monohydrate), commercial cerelose.

† Extracted twice with ethyl alcohol and twice with diethyl ether.

‡ Hegsted *et al.* (4).

§ Wilson's whole liver powder added at the expense of the ration not fat.

tose losses were obtained. The dietary lactose level was then dropped through steps of 40, 32, 24, and 16 per cent, each level being fed until

constant excretions of galactose were secured. In each case the remainder of the 48 per cent of carbohydrate was made up of glucose. Table IV shows the lactose (and equivalent galactose) levels fed, and the per cent of the ingested galactose excreted in the urine.

Decreasing the lactose level from 48 to 40 per cent reduced the urinary loss of galactose in each butter fat group from an average of 15.5 to 7.9 per cent. Lowering the lactose level to 32 per cent caused a still further drop in the amount of galactose lost. Butter fat was slightly less effec-

TABLE IV

Results of Experiments with Synthetic Rations Containing Lactose

Each figure represents the average of three male rats.

Ration		Lactose* in ration								
		48 per cent		40 per cent		32 per cent		24 per cent		16 per cent	
Vitamin level	Fat, 28 *per cent*	Galactose ingested	$\frac{Ex.\dagger}{In.} \times 100$	Galactose ingested	$\frac{Ex.}{In.} \times 100$	Galactose ingested	$\frac{Ex.}{In.} \times 100$	Galactose ingested	$\frac{Ex.}{In.} \times 100$	Galactose ingested	$\frac{Ex.}{In.} \times 100$
		gm. per day		*gm. per day*		*gm. per day*		*gm. per day*		*gm. per day*	
Medium	Butter fat...	2.85	18.3	1.80	8.1			1.47‡	4.68‡		
		1.72	14.2			1.33	5.8			0.80	6.48
	Corn oil.....	1.40	10.4	1.40	3.6						
		1.20	14.4			0.86	7.4				
High	Butter fat...	1.99	12.7	1.70	7.7			1.14	2.43		
		2.35	11.6			1.69	5.2			0.57	4.15
	Corn oil.....	2.66	4.8	1.76	4.4						
		2.57	12.7			1.21	3.7				

* The per cent of galactose furnished by each diet equals one-half the per cent of lactose.

† $\dfrac{\text{Gm. galactose excreted}}{\text{Gm. galactose ingested}} \times 100$.

‡ Average of two male rats.

tive than corn oil at the higher lactose levels. The significance of the slightly lower excretions on the high vitamin B rations must await further study.

Studies with Galactose Synthetic Diets—The influence of fat on the urinary excretion of galactose by animals fed diets containing galactose as such was studied with the rations given in Table V. The level of the B vitamins in these rations was that given in Table III under the "medium" vitamin ration. The fat-soluble vitamins were incorporated into the fat as described previously. Groups of three rats each were placed on Rations

G-2, G-3, and G-4 at galactose levels of 70, 24, and 33.3 per cent, respectively. After constant urinary galactose losses were obtained, the galactose level of each diet was lowered to the next per cent with a correspond-

TABLE V

Composition of Galactose Synthetic Rations

Component	Ration G-1	Ration G-2	Ration G-3	Ration G-4
	per cent	*per cent*	*per cent*	*per cent*
Butter fat	0	10	28	40
Carbohydrate	80	70	48	33.3
Casein	16.7	16.7	20	22.2
Salts 4	3.3	3.3	4	4.5
Liver concentrate*	2.0	1.0	1.0	1.0

Composition of carbohydrate portion

| $\dfrac{\text{Galactose†}}{\text{Glucose‡}}$ | $\dfrac{6}{74}, \dfrac{12}{68}, \dfrac{24}{56}$ | $\dfrac{70}{0}, \dfrac{24}{46}, \dfrac{12}{58}, \dfrac{6}{64}$ | $\dfrac{24}{24}, \dfrac{16}{32}, \dfrac{12}{36}, \dfrac{6}{42}, \dfrac{33.3}{0}, \dfrac{24}{9.3}$ | $\dfrac{16}{17.3}, \dfrac{12}{21.3}, \dfrac{6}{27.3}$ |

* Wilson's 1:20 liver concentrate; added at the expense of the ration not fat.

† *d*-Galactose (anhydrous) practical, Pfanstiehl.

‡ *d*-Glucose (anhydrous) c.p., Merck.

TABLE VI

Results of Experiments with Synthetic Rations Containing Galactose

Each figure represents the average of three male rats.

Galactose in ration	Fat in ration							
	0 per cent		10 per cent		28 per cent		40 per cent	
	Galactose ingested	$\dfrac{\text{Ex.}}{\text{In.}} \times 100$*	Galactose ingested	$\dfrac{\text{Ex.}}{\text{In.}} \times 100$	Galactose ingested	$\dfrac{\text{Ex.}}{\text{In.}} \times 100$	Galactose ingested	$\dfrac{\text{Ex.}}{\text{In.}} \times 100$
per cent	*gm. per day*		*gm. per day*		*gm. per day*		*gm. per day*	
6	1.82	24.1	1.35	18.1†	1.33	9.63†	1.07	7.62
12	2.09	51.8	1.46†	25.4†	0.93	23.2	2.21†	19.2†
16	2.35	46.1	1.86	35.6	1.45	33.5	0.97	22.9
24			3.39	57.7	1.87	53.5	1.41	42.1
33.3							2.46	49.4
70			15.4	68.7				

* $\dfrac{\text{Gm. galactose excreted}}{\text{Gm. galactose ingested}} \times 100$

† Average of two rats.

ing increase in glucose, as shown in the appropriate column of Table V, and the sugar excretion was again followed until constant results were obtained. This process was repeated until all of the galactose levels

shown had been studied. The rats on Ration G-3 were then placed on the fat-free ration, G-1, at a galactose level of 6 per cent. Excretion studies were carried out as before, but in this case the changes in dietary galactose concentration were in an upward direction. The results are given in Table VI.

Fat decreased the loss of galactose, but not to the same extent as when the dietary galactose was furnished in the form of lactose. At the lower galactose levels the fat effect was greater than at the higher levels. There is an approximate linear relationship between the loss of galactose and the per cent of galactose in the diet.

DISCUSSION

The results reported in this paper confirm the conclusion of Schantz, Elvehjem, and Hart (1) that fat increases the utilization of galactose by the albino rat. The latter investigators studied this phenomenon almost exclusively on a liquid skim milk basal ration, and found not only that various fats such as butter fat, coconut oil, etc., were effective, but also that the fatty acid fraction was the active component of the fat. Through the use of various synthetic triglycerides it was shown that only the even numbered fatty acids above 10 carbon atoms could function in lowering urinary galactose loss. Glucose was found to be slightly effective with some of the animals.

Our studies with liquid skim milk diets are in close agreement with those of Schantz *et al.* Animals fed 80, 100, or 150 cc. of mineralized skim milk excreted in the urine an average of 27.1, 23.5, and 26.5 per cent, respectively, of the ingested galactose. The addition of approximately 4 per cent of butter fat to the skim milk lowered the galactose losses to 3.86 and 2.93 per cent, respectively, when 80 or 100 cc. were consumed. From these and other data presented in Table I the following points are evident: (a) fat in some way influences galactose utilization by the rat, (b) the per cent of the ingested galactose excreted in the urine is not dependent on the amount of ration consumed, (c) the amount of galactose actually used by the rat is greatly affected by the total amount of galactose ingested, and (d) the large amount of water necessarily consumed on a liquid milk diet has little apparent influence on the galactose excretion (compare Groups III to VI).

The data given in Table II demonstrate that the addition of glucose to skim milk lowers the galactose excretion, but not to the same extent as does fat, although 9 gm. of glucose would furnish nearly the same number of calories as 4 gm. of fat. An obvious effect of the added glucose is to decrease the dietary lactose concentration from about 45 to about 25 per cent on a dry basis. Since the data in Tables IV and VI show that the

loss of galactose is dependent to a large extent on the per cent of galactose in the diet, it would be expected that 4 gm. of fat would be less effective than the 9 gm. of glucose; however, such was not the case. It is interesting to note that in 1922 Folin and Berglund (7) found that with a human subject 100 gm. of glucose greatly reduced the urinary loss of sugar which resulted from the ingestion of 100 gm. of galactose.

Zialcita and Mitchell (3) reported that fat had no influence on galactose metabolism. Using a ration almost identical to the synthetic ration containing 48 per cent of lactose and the medium level of B vitamins given in the present paper, they found that galactose losses amounted to about 30 per cent when the fat portion of the diet was composed of either butter fat or glucose. When corn oil was used, the value dropped to 20 per cent. From these results the authors concluded that Schantz *et al.* were in error, and that possibly corn oil contained a substance in the non-glyceride fraction which had an effect on galactose metabolism. In support of the former conclusion Zialcita and Mitchell (3) state, "In the present study an artificial solid milk diet was employed. The amounts of fat or glucose, and the lactose involved were about the same (as milk) when converted to a dry basis." This statement is not substantiated by the accepted average composition for milk solids; namely, lactose 36, fat 29, protein 28, and ash 7 per cent. Table IV shows that dropping the dietary lactose level from 48 to 40 per cent was sufficient to cause a decrease in urinary galactose loss from an average of 19 down to 9 per cent.

The lower excretions obtained on corn oil as compared to butter fat by Zialcita and Mitchell, and to a somewhat lesser extent by the present authors, find an explanation in the work of Schantz *et al.* The latter workers found the fatty acids below 12 carbon atoms ineffective in preventing galactose excretion; therefore, it is to be expected that an oil such as corn oil, which has no fatty acids below this limit, would be more effective than one such as butter fat which contains them to the extent of about 9 per cent.

In their study, Zialcita and Mitchell supplemented the rats receiving the glucose ration with "proper amounts of a glucose solution" in order to furnish as many calories as the animals on the fat-containing rations received. This would decrease the per cent of lactose in the animals' daily diet, and might explain their obtaining approximately equal excretions on glucose and butter fat rations.

A comparison of the results on the "medium" and "high" B vitamin diets shows somewhat lower average excretions in the latter case, but further work is necessary before a definite conclusion can be drawn.

The results in Table VI indicate that fat also lowers galactose excretion when free galactose instead of lactose is used in the ration. Within cer-

tain limits the per cent of the ingested galactose in the urine is in an approximately linear relationship with the per cent of galactose in the diet on any level of fat studied. As pointed out earlier in the discussion, the per cent of the galactose excreted is not dependent upon the total amount of ration consumed; however, the amount of galactose actually metabolized by the rat depends upon the amount of galactose ingested.

Dominguez and Pomerene (8) injected galactose intravenously into dogs and studied the plasma concentration and urinary excretion of this sugar. They found that the rate of excretion was proportional to the plasma concentration, but that the constant of proportionality was independent of the quantity injected. However, the rate of utilization was linearly related to the plasma concentration. It would be of interest to determine what effect fat would have on results obtained in this manner.

SUMMARY

1. Fat increases the utilization of galactose by the rat when either lactose or galactose is ingested. This phenomenon occurs on milk or synthetic type rations.

2. The per cent of the ingested galactose lost in the urine is independent of the actual amount of galactose ingested, but is dependent on the per cent of galactose in the ration.

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THE EFFECT OF PROTEOLYTIC ENZYMES ON RAW AND HEATED CASEIN

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It has previously been observed (Greaves, Morgan, and Loveen (1), Morgan (2)) that the heating of dry casein at 140–200° only slightly lessens the digestibility of the protein, but considerably impairs the nutritional value for rats when fed at 18 per cent or lower protein levels. They found that the supplementation of the diet with 0.2 per cent lysine overcame this deficiency and attributed the reduction in growth to injury of the lysine present, with the possibility that histidine might also be involved to a lesser extent. However, chemical analysis of heated casein (3, 4) detected no decrease in the amount of lysine present. The change produced by heat appeared to be in the arrangement or availability of the amino acids, but a definite answer to the question of the cause of lowered biological value has not been forthcoming.

The previous studies were made *in vivo*. With the development of a rapid, accurate method for lysine analysis (4, 5) it was of interest to determine whether *in vitro* studies with crystalline enzymes would show lessened activity of the heated protein and a defective release of lysine during digestion. Consequently, the present study was undertaken with the crystalline enzymes, pepsin, trypsin, and chymotrypsin; the effect of crude pancreas on the raw and heated casein was also investigated for comparison.

Materials

Borden's edible casein, 50 gm., was spread out in a 6 inch Petri dish raised from the oven floor to prevent scorching and heated at 150° for 70 minutes. This material was used throughout the experiments as the source of heated casein.

Crystalline pepsin was prepared from pepsin (Parke, Davis and Company) 1:10,000 by the method of Philpot (6) and the salt-free product was found to contain 22.4×10^{-4} unit per mg., as was found by Northrop (7). Crystalline trypsin and chymotrypsin were prepared from beef pancreas by the method of Northrop and Kunitz (8). The activities were measured by a procedure similar to that of Anson (9). The trypsin contained 55 per cent salt and had 0.44 unit per mg.; the chymotrypsin had 30 per cent salt and 1.4×10^{-2} unit per mg.

Raw beef pancreas, which was obtained fresh from the slaughter-house, was freed of fat, ground in a meat grinder, and used as such.

Lysine decarboxylase was prepared from cultures of *Bacterium cadaveris*, according to the method of Gale and Epps (5) as modified by Zittle and Eldred (4).

Methods

The general procedure for the enzymatic hydrolysis was as follows.

4 gm. of raw casein and 4 gm. of heated casein were each suspended in 80 cc. of water, and the pH was adjusted to 1.8; 40 mg. of crystalline pepsin were then added to each flask, and, after addition of toluene and chloroform, they were incubated 3 or 5 days at 37°. The pH was readjusted to 7.8, 10 mg. of crystalline chymotrypsin and 18.4 mg. of crystalline trypsin were added, and incubation was continued for an equal length of time. The flasks were then heated for 1 hour on the steam bath, centrifuged, and the supernatant fluids were analyzed for total and amino nitrogen, and for lysine.

In two experiments (Nos. 4 and 5) 5 times the above amounts of enzymes were used, with a total digestion period of only 2 days. When fresh pancreas was used, 1 gm. of the ground material was added to the casein suspensions at pH 8.0. Digestion was carried out for 25 to 26 hours. Total and amino nitrogen determinations were carried out by the micro-Kjeldahl and Van Slyke methods.

The procedure for the analysis of lysine was as described by Zittle and Eldred (4) and consisted of the determination of the CO_2 released in the Warburg apparatus by a standard decarboxylase suspension in phosphate buffer at pH 6.0. In a typical analysis, the flasks contained 2.0 cc. of 0.2 M phosphate buffer, 0.5 cc. of heated or raw casein digest adjusted to pH 6.0, and 0.5 cc. of lysine decarboxylase suspension (10 mg. in 1 cc. of water) in the side arm. Control flasks were used for the determination of both the initial and the final dissolved CO_2 in the digests and enzyme, by addition of 0.5 cc. of 2 N H_2SO_4. The experiment was run 40 minutes at 30° in air.

Results

The percentages of amino nitrogen liberated by enzymatic digestion of the raw and the heated casein were compared.

The available lysine was similarly calculated on an equal nitrogen basis from the ratios of the percentage of lysine liberated respectively from the heated and raw casein samples. The percentage of lysine liberated was determined in relation to the total protein per cc. The factor used for converting total N to protein was 6.42 and was calculated from the analysis

of Borden's edible casein, containing 14.15 per cent N, 1.75 per cent ash, and 7.28 per cent moisture.

Table I gives the total nitrogen, amino nitrogen, and lysine contents of the digests from five experiments with crystalline enzymes. The last two columns show the calculated per cent digestibility and available lysine of the heated casein samples, compared with the raw casein control. Table

TABLE I

Digestion with Crystalline Enzymes

Experiment No.	Time	Substrate, casein	Total N	ꞏNH₂-N	Lysine		Digestion, heated / raw	Lysine, heated / raw
	days		*mg. per cc.*	*per cent*	*mg. per cc.*	*per cent*	*per cent*	*per cent*
1	6	Raw	5.0	21.6	0.38	1.19		
		Heated	5.8	17.5	0.32	0.86	81.0	72.3
2	10	Raw	6.54	29.9	1.26	3.00		
		Heated	6.84	27.5	1.00	2.29	92.0	76.3
3	6	Raw	6.79	18.9	0.34	0.78		
		Heated	7.05	18.2	0.22	0.49	96.3	62.8
4	2	Raw	6.39	22.6	0.42	1.02		
		Heated	6.33	20.6	0.27	0.66	91.2	64.7
5	2	Raw	6.22	22.2	0.52	1.31		
		Heated	6.79	20.5	0.42	0.97	92.3	74.0

TABLE II

Digestion with Pancreas

Experiment No.	Time	Substrate, casein	Total N	NH₂-N	Lysine		Digestion, heated / raw	Lysine, heated / raw
	hrs.		*mg. per cc.*	*per cent*	*mg. per cc.*	*per cent*	*per cent*	*per cent*
6	26	Raw	5.69	35.8	1.53	4.20		
		Heated	6.02	31.2	1.21	3.12	87.0	74.5
7	26	Raw	7.55	31.1	0.91	1.88		
		Heated	6.80	28.6	0.73	1.68	92.0	89.4
8	25	Raw	7.08	27.4	0.59	1.30		
		Heated	7.12	25.0	0.29	0.63	93.6	48.8
9	25	Raw	7.35	31.3	1.76	3.74		
		Heated	7.64	28.9	1.38	2.82	92.0	75.5

II gives similar values for four experiments with fresh pancreas. All the values given are the averages of duplicate determinations.

DISCUSSION

It is apparent that digestibility of the heated casein is only slightly impaired, which corroborates the work of Greaves, Morgan, and

Loveen (1). On the other hand, the amount of free lysine released by the enzymes is much lower after heating the casein, although the total amount present in the acid-hydrolyzed protein was found to be nearly the same in the raw casein (7.5 per cent) and the heated casein (7.7 per cent). It is therefore plain that a change in the availability to the action of proteolytic enzymes has occurred during the heating. This may be due to an alteration in the form of linkage.

The enzyme lysine decarboxylase used for analysis has been shown to be very specific (10) and will not attack α-acetyl-, α-methyl-, ϵ-acetyl-, or ϵ-methyllysine or piperidine α-carboxylic acid. A change involving the ϵ- or α-amino group of lysine would therefore affect its availability to this enzyme. The α-amino and carboxyl groups are presumably bound up in the peptide linkages, which remain unaffected by heat, as shown by digestion with the crystalline enzymes. Therefore the ϵ-amino group would appear to be the one involved.

Neuberger and Sanger (11) have studied the availability of several lysine derivatives for the growth of rats and conclude that, although the easily hydrolyzed ϵ-N-acetyllysine is nutritionally adequate, ϵ-carbobenzoxy-lysine is not. The possibility exists, therefore, that a union of the ϵ-amino group of lysine with some other group has taken place with the formation of a linkage not affected by proteolytic enzymes.

Adamson (12) has also shown that heating dl-lysine produces 40 per cent dl-3-aminohomopiperidine, with a free α-amino group available for linkage. This indicates that the ϵ-amino group of lysine enters easily into other linkages at elevated temperatures.

SUMMARY

The combined action of crystalline pepsin, trypsin, and chymotrypsin on raw and heated casein has been studied. Digestion of the heated substrate did not differ greatly from that of raw casein, but the available lysine, as determined by the specific enzyme lysine decarboxylase, was appreciably less.

Similar results were obtained with crude pancreas as the source of proteolytic enzymes.

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THE INFLUENCE OF DIET ON THE RIBOFLAVIN METABOLISM OF THE RAT*

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The dependence of the riboflavin requirement of the albino rat on diet is now well established. Mannering, Lipton, and Elvehjem (1) found that the fat content of the diet exerts a definite influence; and in a later publication Mannering, Orsini, and Elvehjem (2) also demonstrated that the kind of carbohydrate fed affects the riboflavin requirement as measured by the growth rate. Sarett, Klein, and Perlzweig (3) found an inverse relationship between urinary riboflavin excretion and protein intake. Unna, Singher, Kensler, Taylor, and Rhoads (4) reported that the riboflavin content of the liver of rats fed low protein diets is diminished, even if the riboflavin intake is a liberal one. In the experiments of Richter and Hawkes (5) riboflavin was shown to stimulate the appetite for fat and protein.

The reports mentioned above indicate the existence of a close relationship between dietetic ingredients and riboflavin requirement and metabolism. We, therefore, undertook experiments in order to obtain further information on the nature of this relationship.

Methods

The rats were taken from our breeding stock, as they reached a weight of about 50 gm. The stock food of the rats after weaning consisted of a mixture of sprouted wheat, oats, and bran, supplemented by milk and vegetables in season.

Under these conditions the riboflavin content of the liver, kidney, and muscle averaged 20, 19, and 3.2 γ per gm. of fresh tissue, respectively, and that of the urine and blood 0.3 and 0.55 γ per cc. respectively. In a large number of animals tested uniform results were obtained.

Diets—The standard diet (designated Diet N) consisted of alcohol-extracted casein 15 per cent, rice flour 71 per cent, olive oil 10 per cent, and salt mixture 4 per cent. In addition each rat received 100 I.U. of vitamin A and 2 I.U. of vitamin D twice a week, as well as 0.5 cc. of a riboflavin-free yeast extract, corresponding to 0.5 gm. of dried yeast, as described in a

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former publication by Kligler, Guggenheim, and Buechler (6). The ribo-flavin content of this diet was negligible (0.1 γ per gm.).   Diet N supplied about 20 per cent of the calories from protein, 60 per cent from carbo-hydrate, and 20 per cent from fat.   By varying the relative amounts of casein, rice flour, and oil the following diets were prepared (the figures express percentages of calories): high protein diet, protein 34, carbo-hydrate 49, fat 20; low protein diet, protein 11, carbohydrate 69, fat 20; high fat diet, protein 20, carbohydrate 40, fat 40; low fat diet, protein 20, carbohydrate 76, fat 2.

Riboflavin in the organs, urine, blood, and feces was estimated according to Snell and Strong (7).   The feces were treated according to the modifi-cation of Strong and Carpenter (8), which involves removal of fatty stimulatory substances by ether extraction.

EXPERIMENTAL

Riboflavin Content of Organs and Urine under Various Dietetic Condi-tions—Large groups of rats (forty to 150) were kept on the five riboflavin-free diets described above.   Each group was subdivided into two to five sub-groups, which received graded amounts of the riboflavin supplement.[1]   This varied between zero and 20 γ, and was given daily *per os*.   Each subgroup consisted of twenty to thirty animals.   Two rats of each subgroup were killed weekly, and the liver, kidneys, blood, and muscle were tested for riboflavin.   Before the animals were killed a 24 hour urine sample was taken and examined also for riboflavin.   Representative data are given in Table I.

It follows from Table I that the riboflavin content of the organs and the urine of rats on Diet N without riboflavin supplement shows a steady decrease.   On a supplement of 5 γ per day a slight decrease in the riboflavin content of liver, kidneys, and urine was evident after 2 to 3 months of observation.   7.5 γ per day sufficed to maintain the riboflavin balance of the organism.   In spite of the apparent metabolic sufficiency this ribo-flavin dosage does not produce optimal growth.   Larger doses (10 and 20 γ per day) cause a steady increase of riboflavin in liver, muscle, and urine. Three points seem to be noteworthy: (1) Kidneys are sensitive to ribo-flavin deficiency, but they appear to be unable to store riboflavin; (2) the riboflavin level of the blood was remarkably steady.   Even in severe deficiency no blood level was under 0.50 γ per cc. and this level was not elevated even after 3 months by a daily supplement of 20 γ; (3) the most sensitive indicators for riboflavin supply seem to be the liver riboflavin and the rate of the excretion of riboflavin in urine.

Rats kept on the low protein diet showed a greatly reduced organ ribo-

[1] We are very much indebted to ASSIA, Ltd., Tel-Aviv, for kindly supplying riboflavin.

TABLE I

Influence of Various Riboflavin Doses on Riboflavin Content of Liver, Kidneys, Muscle, and Urine of Rats Fed Normal, Low Protein, High Protein, Low Fat, and High Fat Diets

Diet	Riboflavin given per day	Wks. on diet	Riboflavin per gm. found in			
			Liver	Kidneys	Muscle	Urine
	γ		γ	γ	γ	γ
Normal.........	0	4	15	14	2.1	0.04
	0	8	10	10	1.9	0.02
	0	12	7	8	1.2	0.02
	5	4	21	20	3.3	0.3
	5	8	20	19	3.3	0.2
	5	12	18	19	3.3	0.2
	7.5	4	20	19	3.4	0.3
	7.5	8	20	20	3.3	0.3
	7.5	12	20	20	3.2	0.3
	10	4	22	20	3.5	0.4
	10	8	24	20	3.8	0.4
	10	12	25	20	4.0	0.4
	20	4	23	20	3.5	0.4
	20	8	27	20	4.4	0.5
	20	12	30	20	4.5	0.5
Low protein......	7.5	4	11	9	1.5	0.5
	7.5	8	7	7	1.2	0.6
	7.5	12	Dead	Dead	Dead	Dead
	20	4	11	9	1.5	0.6
	20	8	7	7	1.1	0.7
	20	12	Dead	Dead	Dead	Dead
High protein.....	7.5	4	17	17	2.9	0.1
	7.5	8	10	8	1.3	0.02
	7.5	12	Dead	Dead	Dead	Dead
	15	4	20	19	3.4	0.3
	15	8	20	20	3.1	0.3
	15	12	20	20	3.3	0.3
Low fat..........	3.5	4	20	19	3.5	0.3
	3.5	8	20	20	3.3	0.3
	3.5	12	20	19	3.4	0.3
	7.5	4	24	20	3.5	0.5
	7.5	8	26	20	4.1	0.5
	7.5	12	27	20	4.3	0.5
High fat..........	7.5	4	7	7	1.0	0.02
	7.5	8	Dead	Dead	Dead	Dead
	20	4	20	20	3.5	0.3
	20	8	20	20	3.4	0.3
	20	12	20	20	3.3	0.3

flavin content. This reduction was independent of the amount of riboflavin administered. On the other hand, the amount of riboflavin which was

excreted in the urine by these rats was increased, the excretion rate rising with the amount of riboflavin given. The organism seems to be unable to maintain a functional level of riboflavin, and even relatively large doses fail to prevent secondary riboflavin deficiency. The rats died of ariboflavinosis after 8 to 9 weeks.

On high protein diet the requirement for riboflavin appeared to be increased. In this diet administration of 15 γ of riboflavin per day maintained the vitamin in the organs and urine at the same levels as did the administration of 7.5 γ per day in diet N.

The fat content of the diet exerted a very significant effect. There appears to be an inverse relationship between the fat content of the diet and the amount of fed riboflavin which maintains the vitamin at a functional level in the organs and a normal concentration in the urine. The riboflavin

TABLE II

Riboflavin Content of Feces from Rats Fed Various Riboflavin-Free Diets for 5 Weeks

| | Diets | | | | |
	Normal	Low protein	High protein	Low fat	High Fat
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Weight of fresh feces, per 48 hrs.......	2.5	2.4	2.9	3.7	1.8
" " dried " " 48 "	0.90	0.83	0.79	1.60	0.92
	γ	γ	γ	γ	γ
Riboflavin per gm. dried feces.........	26.4	30.1	14.45	22.0	10.3
" excreted in 48 hrs..........	23.76	25.0	11.3	35.2	9.48

levels which could be achieved on Diet N by 7.5 γ per day were only obtained on high fat diet with 20 γ and on low fat diet with 3.5 γ per day.

It seems, therefore, that the protein and fat content of the diet have a pronounced influence on the riboflavin requirement of the rat.

Intestinal Synthesis of Riboflavin under Various Dietetic Conditions—We fed our different diets for 5 weeks without supplementing them with riboflavin. Then the feces of the rats were collected, weighed, and their riboflavin content determined. The average figures obtained in four experiments are given in Table II.

As can be seen from Table II, the daily riboflavin excretion was diminished in the groups on high protein and high fat diets and was increased in the groups on the low fat diet. The excretion in the low protein diet group resembled that in the Diet N group. The reduced riboflavin excretion in the high protein and high fat diet groups and the high excretion in the low fat diet group are correlated to the respectively relative low and high levels of riboflavin in the organs and urine of rats on these diets. It

is noteworthy that with low protein diets no such correlation could be found. These results corroborate our conclusions, drawn from the results given in Table I, that a low protein diet reduces the ability of the organism to store riboflavin and thus increases the excretion in the urine.

The difference in feces riboflavin content between rats fed our different diets finds its explanation in the different rates of intestinal synthesis of the vitamin in the different groups. Support for this explanation was obtained by counts of the number of viable bacteria in the feces and by tests of their riboflavin-synthesizing ability. Feces of the rats kept on our different diets were collected and the numbers of viable bacteria per gm. of fresh feces were determined. The average figures of four experiments are given in Table III.

<center>TABLE III</center>

Number of Fecal Bacteria of Rats Fed Various Riboflavin-Free Diets for 5 Weeks

	No. of bacteria in millions		
Diets	Per gm. fresh feces		Per 48 hrs.
	Mean	Probable error	
Normal......	318	±6.8	795
Low protein.	339	±12.05	804
High " .	116	±8.2	336
Low fat......	450	±4.4	1665
High "	102	±7.7	184

Probability that differences in the above data are due to chance: normal diet *versus* low protein diet, 0.2; normal diet *versus* high protein diet, <0.01; normal diet *versus* low fat diet, <0.01; normal diet *versus* high fat diet, <0.01.

It follows from Table III that the low protein diet does not cause any significant change in the number of viable bacteria in the feces. High protein and high fat diets diminish significantly the number of viable bacteria; low fat diet increases their number. Thus, the riboflavin content of the feces of rats kept on our different diets is seen to be a function of the numbers of intestinal bacteria and of the amount of riboflavin which they synthesize.

In order to strengthen the above conclusion we carried out measurements of the amounts of riboflavin which the intestinal flora of rats fed our different diets can produce. 10 cc. of glucose broth were inoculated with 0.05 cc. of a suspension of feces diluted 1:500.000. The cultures were incubated at 37°. After 24 hours the bacteria were removed by centrifugation and the riboflavin in the supernatant fluid and in the sediment was determined. The cellular sediment was disrupted by repeated freezing

and thawing. In this way we measured the amount of the intracellular riboflavin, as well as the amount excreted into the surrounding medium (Table IV).

As can be seen from Table IV, bacteria of the feces of rats fed the low protein diet produced in 24 hours about the same amount of riboflavin as bacteria from rats fed Diet N. Bacteria from rats fed the high protein and high fat diet produced less riboflavin; but bacteria from rats kept on the low fat diet showed a markedly higher production of riboflavin. About 90 per cent of the riboflavin produced by the bacteria was extracellular. The synthesized vitamin is, therefore, accessible to the rat organism.

TABLE IV

Riboflavin Produced by Intestinal Bacteria of Rats Fed Various Riboflavin-Free Diets for 5 Weeks

Diets	Riboflavin per 10 cc. broth	
	Extracellular	Intracellular
	γ	γ
Normal....................................	6	<1
Low protein..............................	7	1 *Ca.*
High "	4	<1
Low fat...................................	12	1.2
High "	1.5	<1

DISCUSSION

The amounts of riboflavin found by us in the organs and urine of rats fed our standard diet and given 7.5 γ of riboflavin per day correspond more or less with values reported in the literature (9, 10, 4, 11). Our results show unequivocally that the protein and fat of the diet determine the riboflavin content of the organs and the excretion of the vitamin in the urine. Our investigations concerned only three organs, *viz.* liver, kidney, and muscle. According to Sure (12), however, the amount of riboflavin in these three organs represents about 75 per cent of the body's riboflavin. Our data appear, therefore, to be representative for the body as a whole.

This dependence of the riboflavin content of organs and of urine on diet may be due to metabolic factors or to differences in the amounts of riboflavin synthesized by intestinal bacteria. It is possible that riboflavin plays an active part in the metabolism of protein and fat. High protein and high fat diets would, therefore, lead to a higher riboflavin requirement. This explanation was suggested with reference to the effect of protein by Sarett *et al.* (3) and Kleiber and Jukes (13). An analogous mechanism has been proposed for the thiamine-sparing action of high fat diets (14).

Differences in riboflavin synthesis are the alternative possibility. Taylor, Pennington, and Thacker (15) and Mitchell and Isbell (16) proved for the rat that riboflavin synthesized by intestinal bacteria contributes to the body's supply of this vitamin. Mannering *et al.* (2) have shown that dextrin and corn-starch stimulate bacterial synthesis of riboflavin in the rat intestine, and reduce the dietary riboflavin requirement. These authors concluded that dextrin and corn-starch probably increase the amount of riboflavin synthesized in the intestine by providing the intestinal bacteria with a medium which is favorable to such synthesis. The supply of these carbohydrates leads to an increase in the number of the microorganisms, or changes the flora to a type capable of producing larger amounts of the vitamin.

It follows from our experiments that metabolic factors and also the intestinal supply determine the riboflavin level of the organs and urine. The metabolic factors are decisive in a low protein diet. Rats on a low protein diet lack the ability to retain riboflavin. They fail to store the vitamin, even if fed relatively large doses of riboflavin, and thus largely excrete the latter in the urine.

. A very different effect is produced by high protein and high fat diets. Rats kept on these diets contain little riboflavin in their organs, but excrete only relatively small quantities of riboflavin in the urine. This condition is correlated and may be due to a diminished synthesis of riboflavin in the intestine. The reverse holds true for rats kept on a low fat diet. It is noteworthy that rats fed 7.5 γ of riboflavin per day in a standard diet maintain the same levels of riboflavin in their organs as rats fed 15 γ per day in a high protein diet or 20 γ per day in a high fat diet, or 3.5 γ per day in a low fat diet. It follows, therefore, that the dietary riboflavin requirement is not the same in all conditions and that it depends on the dietetic constituents.

SUMMARY

1. The riboflavin content of the liver, kidneys, muscle, and urine of rats kept on the different types of diet ("normal," low protein, high protein, low fat, and high fat) and given graded amounts of riboflavin has been determined.

2. In a "normal" diet a daily riboflavin supplement of less than 7.5 γ caused a steady decrease, whereas 7.5 γ per day maintained, and more than 7.5 γ per day increased, the body's riboflavin level.

3. Rats kept on a low protein diet showed a relatively large excretion of riboflavin in the urine, and were unable to retain riboflavin in their organs.

4. Rats kept on high protein and high fat diets needed at least twice as much riboflavin as rats kept on the "normal" diet for the maintenance in

the organs and urine of an equal level of riboflavin. Rats on low fat diets needed only about half as much riboflavin.

5. The different requirements for dietetic riboflavin of rats kept on different diets have been shown to be due to differences in the amounts of riboflavin which are synthesized in a form available to the organism in their intestines.

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RADIOIODINE AND IODINE FRACTIONATION STUDIES OF HUMAN GOITROUS THYROIDS*

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Certain forms of human hyperthyroidism and myxedema have been attributed to variations in pituitary activity. Medical opinion, however, regards pituitary influences alone as unable to account for the great variety of conditions found in the abnormal thyroid gland. This opinion is supported by the present investigation, since factors such as the level of blood iodine and local conditions within the gland were found to affect thyroid behavior without direct intervention of the hypophysis.

The original purpose of this work was to investigate the metabolism of radioiodine in human thyroids removed at operation, and especially in nodular goiters. The behavior of nodules and surrounding thyroid tissue was compared in regard to iodine content and to the entry of radioiodine into the various iodine fractions. Furthermore, the incidental observation of a low iodine uptake by the gland in a patient with a high blood iodine prompted the systematic investigation of iodine metabolism in dogs with a very high blood iodine.

The last question raised was whether the variety of thyroid responses revealed by chemical analysis would correspond to various locations of the iodine entering the gland. A partial answer to this question was furnished by the autographic (1)[1] study of the extirpated thyroids.

Methods

Prior to thyroidectomy several determinations of the basal metabolism were made. The iodine content in the acetone-insoluble and acetone-soluble fractions of the blood was determined on several occasions in each patient by the methods of Davison and Curtis (2). A solution of radioiodine containing from 25 to 1000 microcuries of the radioelement I^{131} and about 2 γ of iodine I^{127} was administered orally 15 to 23 hours before thyroidectomy, with the exception of Case 17 in which 40 hours intervened between administration and operation. The small iodine content of the radioiodine solution insured that a true picture of the physiological be-

[1] Leblond, C. P., Fertman, M., Puppel, I. D., and Curtis, G. M., *Arch. Path.*, in press.

havior of iodine would be given by the results obtained with radioiodine. After the operation the weight of thyroid tissue removed was recorded and an approximate estimation of the remaining thyroid tissue was made, so that some idea of the total thyroid weight was gained by this method. A careful separation of the nodules from the paranodular tissue was carried out in all cases except in Case 15, in which there was a diffuse goiter. By use of both nodules and surrounding tissues, determinations of I^{127} and I^{131} were made on the various iodine fractions.

The metabolism of radioiodine was also examined in a series of dogs showing a high blood iodine. These animals had been fed a diet of dog biscuits containing 4 mg. of iodine per 100 gm. and of frozen meat containing 0.017 mg. of iodine per 100 gm. The inorganic iodine in the blood of these dogs varied from 0.1 to 0.5 mg. per 100 cc., which values were approximately 100 times those found in men or in dogs fed a diet with an ordinary iodine content.

Although the six dogs investigated had undergone a unilateral thyroidectomy 2 months before the present investigation, this operation should not have affected the results discussed here. The six dogs were given injections of radioiodine I^{131} without carrier and were sacrificed either $\frac{1}{2}$ hour (Dogs A and B), 24 hours (Dog C), 48 hours (Dog D), 72 hours (Dog E), or 94 hours (Dog F) later. The thyroids were examined for the content of I^{127} and I^{131} in the various fractions.

The inorganic iodine, the diiodotyrosine iodine, and the thyroxine iodine were separated according to a method (3) which is a combination of the technique devised by Gutman and coworkers for inorganic iodine fractionation (4) and that devised by Blau (5) for thyroxine separation. The fresh tissue was trimmed, cut in small pieces, and placed on a glass slide in a Fisher-Abderhalden drier at 79° for about 2 hours at normal pressure, then for another 2 hours under the low pressure from a Hyvac pump. The gland was removed, homogenized in a mortar, and returned to the drier for another 2 hours. A second grinding was sometimes necessary at this stage. Four aliquots of the powdered thyroid were then weighed, two smaller ones (about 50 mg. each) for duplicate estimation of the total thyroid iodine and two larger ones (about 200 mg. each) for duplicate fractionation.

The separation of *inorganic iodine* was carried out by shaking the larger aliquots with 30 cc. of water for 2 hours, and then centrifuging for 30 minutes at 2000 revolutions per minute. After the supernatant fluid was separated, the residue was mixed with about 10 cc. of water in the centrifuge tube. After another centrifugation, the two supernatant fluids containing the inorganic iodine were distilled, previous to iodine titration, as described below.

The residue obtained on centrifugation was placed in a 125 cc. Erlenmeyer flask with 16 cc. of water used to wash the centrifuge tube in 2 cc. portions. 1 gm. of barium hydroxide previously kept in a well stoppered bottle was then added. The mixture was heated to gentle boiling by keeping the bottom of the flask slightly above an electric hot-plate. The neck of the flask was connected to a reflux condenser. This hydrolysis was continued for 6 hours. The contents of the flask were then poured into a 125 cc. pear-shaped separatory funnel. The sides of the flask were washed successively with three portions of 1 cc. of distilled water, 1.5 cc. of 10 per cent hydrochloric acid, 0.5 cc. of butyl alcohol, and again with two 1 cc. portions of distilled water. All washings were poured into the funnel.

To the funnel 0.2 to 0.3 cc. of brom-cresol green was then added and the solution was titrated with a 1:1 solution of hydrochloric acid to a yellow color, the acid being added dropwise. Final adjustment to a pH between 3.5 and 4.0 was carried out outside with brom-phenol blue and the same solution of acid. The solution was then shaken with 20 cc. of butyl alcohol for 2 to 3 minutes, and allowed to stand for 1 to 2 hours (or overnight).

The aqueous layer was drawn off into a Kjeldahl flask, the scum being left. 15 cc. of 4 N sodium hydroxide with 1 per cent sodium carbonate were added. The solution was shaken again and allowed to stand for 1 or 2 hours. The aqueous layer was drawn off with the scum and added to the first aqueous solution. The barium precipitate was eliminated by centrifugation without significant loss of iodine, provided it was washed with 5 cc. of water once. In the meantime, the butyl alcohol extract was poured into a Kjeldahl flask. The funnel was washed with 2 cc. of the same alkaline solution as before and 2 cc. of butyl alcohol. Again the aqueous layer was drawn off into the flask containing all the previous water extracts. The iodine found in the pooled solutions was called the *diiodotyrosine fraction*. The butyl alcohol was added to the flask containing the first butyl alcohol extract, the iodine estimated in this extract being the *thyroxine fraction*. The amount of fluid was too large in any fraction for carrying out the oxidation preliminary to iodine distillation. The volume was reduced by gentle boiling. During the evaporation of the inorganic or thyroxine fraction, 2 cc. of the usual alkaline solution were first added.

The oxidation and distillation of iodine were carried out according to the method of Matthews, Curtis, and Brode (6) with the difference that a solution of sodium instead of potassium hydroxide was used to collect the distilled iodine. The radioactive measurements of I^{131} were carried out at this stage by bringing up the volume of the solution to 10 or 25 cc. and taking 2 cc. for the Geiger counter determination. The rest of the solution was used for the estimation of the total iodine I^{127} by the standard thiosulfate method (6).

The radioactivity was expressed as percentage of the injected dose per 100 gm. of dry thyroid tissue; and the total iodine, in mg. per 100 gm. of dry thyroid tissue. The *specific activity* of each thyroid fraction was obtained by calculating the ratio of I^{131} over I^{127} expressed as indicated. The *relative specific activity, i.e.* the ratio of the specific activity of each thyroid fraction to the specific activity of the whole thyroid, is reported in Table I.

In a few cases the inorganic fraction of the blood taken at the time of operation was separated by the method of Davison and Curtis (2), and the radioactive and total iodine were estimated as usual.

Autographic studies (1)[1] were made of fragments of the extirpated thyroids of a few of these patients.

Chemical Results

Comparison of the amount of radioiodine (I^{131}) fixed by the nodules and by the gland showed that in all cases the nodular tissue fixed less radioiodine than the paranodular tissue. The difference was greatest in one case (No. 10) in which the nodule was of the fetal adenoma type with solid trabeculae of thyroid tissue and only a small number of minute follicles. The percentage difference was one of the smallest in Case 17 in which the colloid accumulation characteristic of the nodule was microscopically quite similar to that of the paranodular tissue. In the three other cases of nodular goiter (Nos. 13, 14, and 18) the nodules were distinctly colloid in appearance, showing on section wet surfaces and unusual fluidity of the colloid, while the paranodular tissue was firmer, more reddish, and did not contain appreciable amounts of fluid.

The estimations of ordinary iodine (I^{127}) in the nodules as compared to the paranodular tissue showed in all cases a smaller amount of iodine in the nodules. It may be noted that, if the results had been estimated on the basis of the fresh weight, the differences would have been much more considerable, since the nodules were richer in fluid material.

Comparison of the specific activities in the nodules and in the paranodular tissue showed that in general the specific activities were smaller in the nodules, indicating that the turnover of iodine was slower in the nodules than in the glandular tissue itself.

The inorganic iodine fractions contained only a very small percentage of the total I^{127} and I^{131}. The percentage of inorganic iodine was usually smaller in the nodules than in the paranodular tissue. Furthermore, in both tissues, the comparison of radioiodine to ordinary iodine showed a greater proportion of radioactivity in the inorganic iodine fractions than in the whole gland, a fact expressed by the high values of the relative specific activity in the inorganic iodine.

The diiodotyrosine iodine fractions, which are really the residual fractions

after elimination of the inorganic iodine and of thyroxine iodine, constituted the bulk of both the radioactive and the total iodine in the gland. Their specific activity was smaller than that of the inorganic iodine.

Finally, the thyroxine iodine fractions contained a variable amount of iodine, smaller in the nodules than in the paranodular tissue. However, the turnover of thyroxine as estimated by the specific activity was rather comparable in both nodular and paranodular tissues.

The values for the blood inorganic iodine showed a higher specific activity than in the inorganic iodine fraction of the thyroid.

In Case 17, operated on 40 hours after administration of radioiodine, most of the features described above could not be recognized. However, the nodules contained less I^{127} and I^{131} than the surrounding thyroid tissue, but the difference was not so marked as in other cases. In addition, the specific activities in the inorganic and diiodotyrosine fractions were of the same order of magnitude. Apparently the delay in operating on the patient had enabled the level of diiodotyrosine to rise up to or above that of inorganic iodine.

In Case 18 the fixation of radioactive iodine was markedly smaller than in the other cases. Attention may be called to the fact that the inorganic iodine content of the blood was one of the highest. The rôle of the high level of blood iodine on iodine fixation by the thyroid was investigated in the dogs given a diet rich in iodine. In these animals (Table II) the percentage of injected radioiodine which found its way into the thyroid gland was still smaller than in Case 18. Thus it may be calculated that in most of the patients about 50 per cent of the injected dose was fixed; in Case 18 about 4 per cent of the injected dose was fixed; and in the dogs approximately 0.5 per cent of the injected dose was taken up, which is much less.

In the dogs, the amount of chemical iodine I^{127} was high in the three fractions when compared with that of the patients (Table I) or with dogs (3) given a diet with ordinary amounts of iodine. The increase was most pronounced in the inorganic and thyroxine fractions.

The specific activities (Table I) showed a predominance of radioactivity in the inorganic fraction soon after the injection, succeeded by a fairly rapid decrease. On the other hand, the radioactivity in the diiodotyrosine fraction after its initial increase appeared stabilized.

Interpretation

The most striking results were those pointing to a less active behavior in the adenomatous formations than in the surrounding thyroid tissue, as shown by a smaller iodine content, a smaller iodine fixation, and a slower turnover of iodine. These differences were most marked in Case 10 in which the gross appearance and histology showed that the nodule was the

TABLE I

Radioiodine and Iodine Studies of Patients with Goiter

Case No.	Type of goiter	Average basal metabolism rate	Approx. wet weight of whole gland (gm.)	Avg. blood iodine Acetone-insoluble (γ %)	Avg. blood iodine Acetone-soluble (γ %)	Site	Total iodine — Per cent of radio-iodine given	Total iodine — Elemental iodine (mg. %)	Total iodine — Specific activity*	Inorganic iodine — Per cent of radio-iodine given	Inorganic iodine — Elemental iodine (mg. %)	Inorganic iodine — Specific activity*	Diiodotyrosine iodine — Per cent of radio-iodine given	Diiodotyrosine iodine — Elemental iodine (mg. %)	Diiodotyrosine iodine — Specific activity*	Thyroxine iodine — Per cent of radio-iodine given	Thyroxine iodine — Elemental iodine (mg. %)	Thyroxine iodine — Specific activity*	Blood (inorganic fractions), per 100 cc. blood — Per cent of radio-iodine given	Blood — Elemental iodine (γ %)	Blood — Specific activity
10	Non-toxic diffuse colloid with fetal adenoma; M. H., 445260	+3	53	1.30	3.58	Nodule	2.7	2.4	0.8 (1.0)	1.5	0.4	3.8 (4.7)	0.9	1.6	0.6 (0.7)	0.6	0.9	0.6 (0.7)			
						Para-nodular tissue	195.2	250.0	0.8 (1.0)	8.3	7.6	1.1 (1.4)	267.2	179.0	0.9 (1.1)	12.9	63.3	0.2 (0.3)			
13	Mildly toxic nodular goiter; L. D., 444662, 445388	+18	133	2.20	2.62	Nodule	16.5	77.0	0.2 (1.0)	3.0	1.0	3.0 (15.0)	11.0	63.2	0.1 (0.9)	2.1	10.0	0.2 (1.0)			
						Para-nodular tissue	92.1	180.2	0.5 (1.0)	7.6	3.5	2.2 (4.4)	15.8	108.2	0.15 (0.3)	50.9	63.3	0.8 (1.6)			
14	Non-toxic nodular goiter; M. M., 445877, 444919	+3	216	1.70	2.88	Nodule	14.0	86.9	0.16 (1.0)	0.35	1.9	0.19 (1.2)	11.4	63.6	0.18 (1.1)	2.1	17.8	0.12 (0.7)			
						Para-nodular tissue	90.0	190.2	0.5 (1.0)	11.1	9.5	1.2 (2.4)	82.8	144.8	0.6 (1.2)	6.7	25.9	0.3 (0.6)			

Note: Specific activity values are given as absolute value (relative value).

No.	Diagnosis					Fraction																		
15	Non-toxic diffuse colloid goiter; E.S., 445276	−7	74	1.02	1.68		163.0	163.0	1.0	**1.0**	13.0	3.5	3.7	**3.7**	137.0	114.0	1.2	**1.2**	16.0	25.0	0.6	**0.6**	0.004	0.603	0.007
17	Non-toxic nodular goiter; R.G., 445841	−20	196	1:07	2.06	Nodule	103.0	66.0	1.6	**1.0**	3.6	2.4	1.5	**0.9**	95.0	51.0	1.9	**1.2**	3.8	9.2	0.4	**0.3**	0.011	0.0046	2.4
						Para-nodular tissue	124.0	113.0	1.1	**1.0**	4.6	2.6	1.8	**1.6**	106.0	80.0	1.3	**1.2**	5.9	20.5	0.31	**0.3**			
18	Non-toxic nodular goiter; S.R., 446693	+4	108	1.70	4.12	Nodule	5.8	160.2	0.03	**1.0**		4.5			4.5	123.0	0.03	**1.0**		34.6			0.144	0.016	9.0
						Para-nodular tissue	12.2	228.0	0.05	**1.0**		7.1			11.0	160.0	0.06	**1.20**		59.2					

* The figures in bold-faced type represent the relative specific activity, the ratio of the specific activity of the thyroid fraction to that of the whole thyroid.

TABLE II

Radioiodine and Iodine Studies of Thyroid Gland of Dogs Receiving High Iodine Intake

Dog	Time after injection	Approximate dry weight of whole gland	Radioiodine and iodine fractionations of thyroid gland, per 100 gm. dry weight													Radioiodine and iodine fractionation of blood (inorganic fraction), per 100 cc. blood		
			Total iodine			Inorganic iodine			Diiodotyrosine iodine			Thyroxine iodine					
			Per cent of radioiodine given	Elemental iodine	Specific activity*	Per cent of radioiodine given	Elemental iodine	Specific activity*	Per cent of radioiodine given	Elemental iodine	Specific activity*	Per cent of radioiodine given	Elemental iodine	Specific activity*	Per cent of radioiodine given	Elemental iodine	Specific activity*
	hrs.	gm.		mg. per cent			mg. per cent			mg. per cent			mg. per cent			γ per cent	
A†	½	0.110	215.0	340.0	0.63 / **1.0**	151.0	40.0	3.77 / **6.13**	52.0	205.0	0.25 / **0.4**	10.0	86.0	0.11 / **0.2**	2.5	0.231	10.9 / **18.17**
B†	½	0.190	320.0	987.0	0.32 / **1.0**	220.0	55.0	3.9 / **12.4**	97.0	589.0	0.2 / **0.5**	11.0	271.0	0.04 / **0.1**	1.9	0.11	17.5 / **58.3**
C	24	0.116	31.8	759.0	0.042 / **1.0**	3.39	46.1	0.074 / **1.75**	26.2	465.0	0.056 / **1.33**	6.2	249.0	0.025 / **0.6**	0.13	0.24	0.56 / **13.3**
D	48	0.195	25.2	820.0	0.030 / **1.0**	2.1	47.2	0.044 / **1.47**	20.6	499.0	0.041 / **1.37**	5.9	269.0	0.022 / **0.73**	0.61	0.20	0.31 / **10.33**
E	72	0.235	35.1	836.0	0.042 / **1.0**	2.0	41.9	0.048 / **1.14**	26.8	482.0	0.053 / **1.26**	7.2	263.0	0.028 / **0.66**	0.45	0.18	0.25 / **5.71**
F	94	0.135	11.2	559.0	0.020 / **1.0**	0.79	37.0	0.021 / **1.05**	8.0	301.0	0.027 / **1.35**	2.2	181.0	0.012 / **0.60**	0.03	0.33	0.10 / **5.0**

* The figures in bold-faced type represent the relative specific activity, the ratio of the specific activity of the thyroid fraction to that of the whole thyroid.

† Dogs A and B were given pituitary extract (Ayerst, McKenna, and Harrison) for 3½ days before autopsy. The thyroid mutation was histologically mild.

typical so called fetal adenoma. Marine (7) observed that fetal adenomas contained rather small amounts of iodine. Wegelin (8) found that fetal adenomas fed to tadpoles exerted less effect on growth than the surrounding tissues. On the other hand, a number of investigators, confusing the parenchymatous appearance of fetal adenomas with that of hyperactive thyroid tissue in which there is also a marked predominance of epithelial over colloid material, were led to believe that fetal adenomas, at least in their trabecular form, were made of hyperactive thyroid tissue. The poor fixation of radioiodine in Case 10 may indicate that the typical fetal adenoma is made of rather inactive thyroid tissue.

The other nodules were quite different from the fetal adenoma, containing large follicles filled with considerable amounts of colloid. Many authors (8) believe, however, that there are no fundamental differences between fetal and colloid adenomas. On the other hand, the colloid nodules behaved like the fetal adenomas in regard to fixation of radioiodine, although in the former differences between paranodular and adenomatous tissue were considerably less pronounced than in the latter. At any rate, the behavior of the adenomas furnished an example of how local factors inside the thyroid may influence iodine fixation.

The second factor which may affect iodine metabolism is the level of the blood iodine, since one patient, as well as the six experimental dogs with a high blood inorganic iodine, showed a small uptake of radioiodine in the thyroid. The first cause of this was probably the dilution of the radioactivity in the inorganic iodine present in the blood, since the mixing of the radioactivity with a large amount of iodine reduced the chances of the radioactive atoms to enter the gland. In addition, there was a definite shift in the iodine metabolism of the thyroid, as shown by the high iodine content of the gland, this high content of iodine corresponding to what was previously called "saturation" of the thyroid (9). Indeed, when a large dose of radioiodine was given a few hours after injection of a large dose of ordinary iodine, the thyroid, being "saturated" by the ordinary iodine, fixed much smaller amounts of radioiodine than usual (9). Similarly the low radioiodine uptake found here, as well as the high iodine and thyroxine content of the thyroid (Table II), was apparently due to some extent to a chronic "saturation" of the gland owing to continuous availability of excessive amounts of iodine from the blood.

The rapidity of the "saturation" produced by an injection of a large iodine dose (9) proved that effects of this type were the result of a direct action on the thyroid. The hypophysis, therefore, did not play an immediate rôle in the production of these results.

In conclusion, studies on iodine metabolism showed three types of influences affecting the thyroid: First, *local factors*, such as the nodules so

common in old age, produced variations of iodine metabolism within the thyroid; second, a high *level of blood iodine* "saturated" the gland with iodine, producing a storage of thyroxine inside the gland (Table II); and third, *pituitary factors*, when excessive or deficient, activated or slowed down the iodine turnover (3). All of these influences may occur in man.

These studies showed in both human patients and experimental animals that during the first 2 days after injection (Tables I and II) the specific activities were higher in the inorganic iodine than in the other fractions of the glands. This was in agreement with the results obtained when a large dose of I^{127} was used to carry the injected radioactive iodine (9). In all these cases the inorganic iodine coming from the blood entered the thyroid as such. However, in another experiment with dogs, given an injection of radioiodine without a carrier, it had been found (3) that the specific activity was highest in the diiodotyrosine fraction as early as a half hour after injection. But the inorganic iodine I^{127} was comparatively higher in those dogs (3) than in the patients examined (Table I). This observation suggested a possible explanation of the discrepancy. There may exist in the thyroid gland two inorganic iodine fractions, one into which the radioactive inorganic iodine entering the gland may diffuse and another separated in a chemical or anatomical way from mixture with the radioiodine. The protected form could be rather small, as in the patients, or great enough, as in the dogs (3), to increase the denominator of the specific activity in inorganic iodine to such an extent that this specific activity would be lower than that of diiodotyrosine iodine. Such a hypothesis also eliminates the need for postulating that the transformation of ionic iodine into diiodotyrosine takes place at the level of the cell membrane (3).

The specific activity in the diiodotyrosine fraction was intermediary between that in the inorganic fraction and that in thyroxine, at least during the first 48 hours. This may indicate that thyroxine was synthesized more slowly than diiodotyrosine. This result was satisfactorily explained along the lines of Harington's hypothesis (10) that diiodotyrosine is the precursor of thyroxine, as shown by previous results (3).

Comparison of the chemical and histological results[1] showed that the radioiodine passed through the cellular epithelium of the thyroid follicle to be localized as diiodotyrosine in the colloid.

SUMMARY

Thyroid adenomas were functionally less active than the surrounding thyroid tissue, as shown by a lower iodine content, a smaller fixation of radioiodine, and a slower turnover of the iodine. The difference was most considerable in one case of so called fetal adenoma, but it was still well demonstrated in four cases of colloid nodules.

The presence of a high level of blood inorganic iodine seemed to produce a large concentration of iodine in the thyroid.   This increase in iodine content appeared distributed in the three thyroid fractions.

The iodine after entering the thyroid gland as inorganic iodine was rapidly transformed into diiodotyrosine and as such was deposited in the colloid of the thyroid follicle.

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THE EFFECT OF RIBONUCLEIC ACID AND ITS HYDROLYTIC PRODUCTS AND OF DESOXYRIBONUCLEIC ACID ON SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE

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The studies of Adler, von Euler, and Skarzynski (1) had shown that yeast adenylic acid and adenosine were inhibitory to succinic dehydrogenase. These observations were confirmed in the present studies with the manometric and methylene blue decolorization techniques. Studies were made of cytochrome oxidase as well. It is tentatively suggested that one of the functions of the nucleic acids and their hydrolytic products may reside in their influence on the enzymatic oxidation-reduction systems. Changes in the ratio of nucleic acid to hydrolytic products in the cell may be of importance because the diffusible nature of the latter extends the range of this influence.

The present studies were initiated when it was observed that ribonucleinase was inhibited by mononucleotides (2) presumably by competition with the substrate.[1] The thought occurred that nucleic acid nucleotides might inhibit enzymes requiring nucleotide coenzymes by competition with the latter. The observations of von Euler *et al.* (1, 3, 4) on the effect of nucleic acid hydrolytic products on dehydrogenases were of interest in this connection. Succinic dehydrogenase (1) was chosen for initial study, even though it does not seem to require a nucleotide coenzyme, because of its relatively simple preparation and because it afforded an opportunity to determine whether the inhibition of this enzyme by ribonucleinase, observed by Potter and Albaum (5), might be explained on the basis of the nucleotides liberated from the nucleic acid which is present (6).

EXPERIMENTAL

Reagents—The enzyme preparations were obtained from pig heart muscle by the procedure of Keilin and Hartree (7) with the exception of the precipitation at pH 4.5. The solid matter content of the preparations was about 2.0 per cent. Six preparations in all were studied. The cytochrome *c* was prepared from horse hearts in the usual way (8, 7). The ribonucleic acid (yeast) and derivatives were commercial products. The mixed mono-

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[1] Further studies have shown that the inhibition is not by competition with the substrate.

nucleotides were prepared by the complete hydrolysis of ribonucleic acid
with NaOH (2) and neutralization with HCl. 1.0 cc. represented approx-
imately 6.8×10^{-5} mole of mononucleotides (from 23 mg. of nucleic acid);
the concentration of NaCl was 0.08 M. The desoxyribonucleic acid was
prepared from calf thymus by the method of Hammarsten (9).

Assay--The manometric determinations of the enzymes were performed
at 37°. The following reagents were used for the succinoxidase system:
2.0 cc. of 0.15 M sodium phosphate, pH 7.2, 0.5 cc. (2.0 mg.; 1.21×10^{-7}
mole) of cytochrome *c*, 0.4 cc. of diluted enzyme (diluted with about 1.5
volumes of the phosphate buffer so that the oxygen uptake with succinate
was approximately 700 c.mm. per hour), and 0.3 cc. of 0.5 M succinate,
pH 7.2. No oxygen was taken up without the substrate or with the enzyme
heated to 100°; the oxygen consumption with the substrate was increased
about 35 per cent by the addition of cytochrome *c*. When solutions of sub-
stances to be studied were added to this system, an equal volume of the
phosphate buffer was displaced; variation of the phosphate in this manner
had no influence on the enzyme. The pH was checked after each experi-
ment and always found to be pH 7.2. The addition of Ca or Al (10) did
not increase the activity of the enzyme. The oxygen uptake was propor-
tional to the amount of enzyme up to 0.2 cc. of the undiluted preparation;
beyond this the diffusion of the oxygen into the reaction mixture became a
limiting factor with our equipment.

For the determination of the cytochrome oxidase the same system was
used, except that 0.2 N hydroquinone replaced the succinate. The oxygen
consumption with the boiled enzyme due to the spontaneous oxidation of
the hydroquinone was on the average 160 c.mm. per hour. The oxygen
uptake of the unboiled active enzyme was increased about 100 per cent
by the addition of cytochrome *c*.

The succinic dehydrogenase was determined by the methylene blue de-
colorization procedure at 32–37° with the above reaction mixture, except
that the cytochrome *c* was omitted and 0.1 cc. of methylene blue (11.2 mg.
per 10 cc.) was added. Decolorization times of 10 to 15 minutes were ob-
tained with the usual technique (11) with 0.1 cc. of the undiluted enzyme;
the percentage of inhibition of added substances was calculated from
$(1/T^0 - 1/T) \times 100/1/T^0$ in which T^0 equals the decolorization time for
the control and T the time with the added substance.

Results

Studies of Succinoxidase System by Manometric Technique—The inhibi-
tory effect of the ribonucleic acid hydrolysate (mixed mononucleotides)
on the succinoxidase system is shown in Fig. 1; the inhibition with enzyme
Preparation III was not quite so great as with Preparations IV, V, and VI.
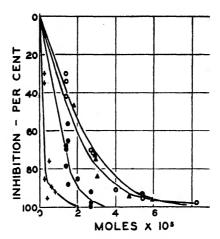
FIG. 1. Effect of ribonucleic acid, ribonucleotides, and desoxyribonucleic acid on the succinoxidase system. The number of moles indicated are contained in the test volume of 3.2 cc. The moles of mononucleotides are calculated from the average molecular weight of 339; the nucleic acids are plotted on the basis of their nucleotide equivalents. O mononucleotides, enzyme Preparation III; ● mononucleotides, enzyme Preparations IV, V, and VI; △ ribonucleic acid, enzyme Preparations IV, V, and VI; + desoxyribonucleic acid, enzyme Preparations V and VI.
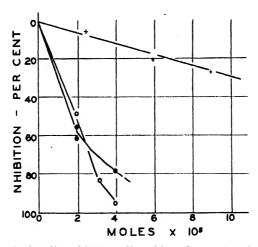
FIG. 2. Effect of adenylic acid, guanylic acid, and pyrophosphate on the succinoxidase system. The number of moles indicated are contained in the test volume of 3.2 cc. O guanylic acid; ● pyrophosphate; + adenylic acid, enzyme Preparation V. With enzyme Preparation II less inhibition was obtained; with Preparation IV more inhibition was obtained; in each case the inhibition was proportional to the amount of adenylic acid.

Guanylic acid was about as inhibitory as the mixed mononucleotides (Fig. 2); adenylic acid (Fig. 2) was much less inhibitory, more variable in its effect with different enzyme preparations, and the curve was dissimilar, being a straight line in the range studied, whereas the above substances gave hyperbolas. Guanosine (4.0×10^{-5} mole) was not inhibitory. Adenosine was not inhibitory when studied manometrically; two commercial preparations (British Drug Houses and Mackay) gave the same negative results. Since Adler, von Euler, and Skarzynski (1) had observed a slight increase in the inhibition obtained with adenosine with the dilution of the substrate, experiments were performed with 0.1 cc. of succinate, but with negative results. Manometric experiments with methylene blue present (3.0×10^{-6} mole) and with the cytochrome system inhibited with cyanide were performed, but here also adenosine had no effect. It will be seen later that with the methylene blue decolorization technique inhibition was obtained in confirmation of the findings of Adler, von Euler, and Skarzynski (1).

Ribonucleic acid was inhibitory, as shown in Fig. 1 where the inhibition is plotted in terms of moles of mononucleotides contained in the nucleic acid; the inhibition on this basis is somewhat less than that obtained with the mononucleotides with the same enzyme preparations; unfortunately the effect of nucleic acid was not tested on enzyme Preparation III. Desoxyribonucleic acid is very inhibitory; in Fig. 1 the effect is plotted in terms of the mononucleotide content.

Other substances were tested which might have a bearing on the interpretation of the data for nucleic acid and products. Pyrophosphate was found to be inhibitory (Fig. 2) in confirmation of the data quoted by Dixon and Elliott (12) for the decolorization of methylene blue. This apparently is a competitive inhibition (13). Sodium chloride in the amount present in the mononucleotides had a negligible effect; 5×10^{-5} mole of $(NH_4)_2SO_4$ had no effect; 1.0×10^{-6} mole of cystine was very inhibitory (30.9 per cent), in confirmation of Potter and DuBois (14). In the experiments described above the substrate was placed in the side arm of the Warburg flasks. With this procedure the enzyme system and inhibiting agent were in contact for 18 minutes before the succinate was added. In other experiments the enzyme was placed in the side arm; identical control oxygen uptake was obtained by this procedure, but no inhibition was obtained with the mononucleotides, ribonucleic acid and adenylic acid, the only nucleic acid substances tested in this manner. Although there was no inhibition immediately, inhibition was noticeable later (in about 10 minutes), as would be expected. It might be mentioned that pyrophosphate was equally inhibitory by either procedure.

The following experiments were performed to decide whether inhibiting

agents were produced from the substances studied by a slow enzyme action (the heart preparation probably contains a variety of enzymes) or whether the inhibition represented a slow chemical action. 5.0 cc. of phosphate buffer, 4.0 cc. of mononucleotides, and 1.0 cc. of undiluted enzyme were incubated at 37° for 30 minutes; the enzymes were destroyed by heating in a boiling water bath for 5.0 minutes. Use of this mixture as a source of mononucleotides gave the same inhibition observed before when the substrate was in the side arm, and no inhibition when the enzyme was in the side arm. This evidence for a slow chemical reaction mixture was confirmed by a similar experiment in which the above reaction mixture was dialyzed 18 hours at 7° against 10 cc. of phosphate buffer. Only about 30 per cent of the expected amount of inhibitor diffused through the cellophane, but this also inhibited only when in contact with the succinoxidase system before the substrate was added. The poor diffusion through the cellophane was due to the presence of the heart tissue complex; alone, the mononucleotides, which are the inhibiting substances, diffuse freely.

Cytochrome Oxidase—The mixed mononucleotides and nucleic acid had little effect on the cytochrome oxidase (less than 10 per cent inhibition for 5.0×10^{-5} mole). The effect of the former appeared to be largely accounted for by the NaCl known to be present.

The succinoxidase system studied above is composed of the enzymes succinic dehydrogenase and cytochrome oxidase. Since cytochrome oxidase is not affected by the nucleic acid compounds, it must be concluded that the inhibition of the succinoxidase system described above represents an effect on the succinic dehydrogenase.

Nature of Inhibiting Substance—The ribonucleic acid, originating in yeast, was suspected of containing sulfhydryl compounds which, if present, would be oxidized to the highly inhibitory disulfide, a transformation observed by Potter and Schneider (10). This might have accounted for the slow action of the inhibiting substances. Utilization of the nitroprusside test (0.5 cc. of substance under test, 1.0 cc. of 1 per cent sodium cyanide, and 2 drops of 2 per cent sodium nitroprusside) showed that although 1.0×10^{-6} mole of cystine gave a strongly positive test, the solutions of ribonucleic acid, mononucleotides, adenylic and guanylic acids, all of which (except the adenylic acid solution) were more inhibitory than the cystine, gave negative tests. No substances preventing the test were present, for on adding 0.5 cc. of cystine (1.0×10^{-6} mole) to the above substances positive tests were readily obtained.

To ascertain whether the inhibitory effect of the nucleic acid was due to the high polymer fraction, portions of mononucleotides (46.0 mg. per cc.) and ribonucleic acid (20.0 mg. per cc.) were placed in cellophane tubes and dialyzed against equal volumes of phosphate buffer for 18 hours at 7°. In

the case of the mononucleotides the inhibiting substance was equally distributed between the inside and outside of the tube, as were the mononucleotides; with the nucleic acid only 7 per cent of the total amount of inhibiting substance had diffused through the cellophane which can be accounted for roughly by the mononucleotides present in this preparation (Sample EA_2 (2)). The above distributions were confirmed by the solid matter content of the inside and outside solutions. Accordingly, it must be concluded that the inhibitory effect of ribonucleic acid is inherent in the high polymer fraction.

Studies of Succinic Dehydrogenase by Methylene Blue Decolorization Technique—The inhibition data by this procedure are given in Fig. 3. Ade-
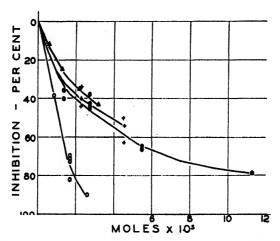
FIG. 3. Effect of adenosine, adenylic acid, and mononucleotides on succinic dehydrogenase (methylene blue decolorization). The number of moles indicated are contained in the test volume of 3.2 cc. + adenosine, present study; △ adenosine, data of Adler, von Euler, and Skarzynski (1); ● adenylic acid; ○ mononucleotides.

nosine is quite inhibitory and to about the same degree as found by Adler, von Euler, and Skarzynski (1); adenylic acid is inhibitory and here the effect gives a hyperbolic curve; the mononucleotides were very inhibitory. Adenosine and adenylic acid gave a greater degree of inhibition by this technique than by the manometric procedure. Since Potter and Schneider (10) had observed that numerous substances were oxidized by a similar enzyme system to give compounds that were inhibitory, there was a possibility that, although sulfhydryl was excluded, other compounds amenable to oxidation might be present. As a check on this possibility the enzyme was placed in the side arm and the system evacuated, which gave anaerobic conditions before the enzyme and inhibitor (mononucleotides) were mixed.

The inhibition obtained with experiments performed in this manner was about as great as when the substrate was placed in the side arm.

DISCUSSION

The nearly equivalent inhibition obtained with equal weights of ribonucleic acid and mononucleotides indicates that from a molecular standpoint the nucleic acid is much more inhibitory than the mononucleotides;[2] this finding suggests also that the inhibition must reside in the mononucleotide radical. This observation did raise the question whether a low molecular weight substance occurring in both might cause the inhibition. Ammonium sulfate was tested, since Greenstein and Chalkley (15, 16) have described tissue enzymes capable of deaminating nucleic acids as well as nucleotides. The inaction of ammonium sulfate and the apparent nonenzymatic nature of the time lag in the effect of the nucleic acid compounds exclude this possibility. Sulfhydryl compounds, likely contaminants in preparations from yeast which is relatively rich in glutathione, are excluded by the negative nitroprusside test. Pyrophosphates were tested, since it was remotely possible that oxidative phosphorylation of the nucleotides might take place, but these are excluded because of the rapid reaction of pyrophosphate as such and the non-enzymatic nature of the time lag. The results with the anaerobic methylene blue technique appear to exclude an oxidative phenomenon. Strong evidence that the source of the inhibition effect is the nucleic acid and the mononucleotides themselves is given by the dialysis experiments; in the case of the latter the inhibitory substance readily dialyzed, in the case of the former the inhibitory substance was non-dialyzable. Further evidence for the conclusion that the inhibition is inherent in the substances themselves is the close agreement with the data of Adler, von Euler, and Skarzynski (1) for adenosine, for it is unlikely that a contaminant would appear in both preparations to the same degree.

Keilin and Hartree (17) have observed that coenzyme I (diphosphopyridine nucleotide) strongly inhibits succinoxidase. This effect, which was found to be due to the ultimate production of the inhibitory oxalacetic acid from succinate, can be excluded in the present case, since the effect they observed occurs slowly after the addition of the succinate. It should be noted that coenzyme II was inhibitory to a much less marked degree and by an unknown mechanism.

Other observations have been made of the inhibitory action of nucleic acid hydrolytic products; in addition to the work of von Euler et al. quoted with succinic dehydrogenase (1), lactic acid dehydrogenase (1, 3) and

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[2] The average molecular weight of the mononucleotides is 339, the molecular weight of ribonucleic acid is about 10,000; in equal weights of these substances the molecular ratio would be about 30:1.

alcohol dehydrogenase (4) were also found to be inhibited. Coenzyme I (diphosphopyridine nucleotide) is a component of these last two enzymes and it might be expected that the ribonucleotides would have a competitive effect, but this does not seem to be the case (3).

Recently Greenstein and Chalkley (18, 19) have reported on the inhibitory effect of ribo- and desoxyribonucleic acids on an unknown tissue dehydrogenase and xanthine dehydrogenase; under some conditions either no effect or stimulation was obtained, depending on the concentration of methylene blue. These workers found that desoxyribonucleic acid was more effective than ribonucleic acid, as has been found in the present studies. The difference in inhibition observed in the present work with the methylene blue decolorization technique and with the manometric technique may be attributable to variation in the concentration of suitable hydrogen acceptors, as found by the above workers. It should be noted in passing that Adler and von Euler (20) found that yeast nucleic acid stimulated a similar enzyme preparation, in their case by a succession of enzymatic reactions which produced xanthine which in turn served as substrate for the xanthine dehydrogenase therein; thymus nucleic acid had no effect.

Whatever the nature of the inhibition caused by nucleic acids and their hydrolytic products which has been observed by various workers, it appears to be real and general for oxidation-reduction enzymes. Future studies will show whether other types of enzymes are affected as well. The observations in the present studies and the similar observations of others suggest a possible important rôle of nucleic acids and their hydrolytic products in their interaction with the dehydrogenase enzyme systems. The mechanism of this interaction and whether it has physiological importance remain for future studies to reveal. Greenstein and Chalkley (19) feel that their observations with dehydrogenases and nucleic acid might provide a clue to some of the mechanism which nuclear (chromosomal) components exert in the maintenance and regulation of cellular functions.

The interaction of both nucleic acid and mononucleotides on succinic dehydrogenase is of great interest and suggests that the ratio of nucleic acid to mononucleotides would be of importance, for the diffusible nature of the latter extends the range of the observed influence of these compounds. It is perhaps of great significance that changes in the ratio or amounts of these substances occur in pathological conditions (*i.e.*, cancer) and accompany irradiation with ionizing rays (x-rays and neutrons).

SUMMARY

Equal weights of ribonucleic acid and hydrolyzed nucleic acid (mono- nucleotides) were about equally inhibitory to succinic dehydrogenase; adenylic acid and guanylic acid were both inhibitory. Inhibition was not

immediate and appeared to be a slow chemical reaction rather than the intervention of an enzyme.

Desoxyribonucleic acid was more inhibitory than any of the above substances. Adenosine, which did not inhibit when tested by the manometric procedure, was inhibitory when tested by the methylene blue decolorization technique. Data obtained indicate that the inhibition was a property of the ribonucleic acid and mononucleotides and not a result of contaminating substances.

A possible important rôle for nucleic acid and its hydrolytic products in relation to oxidation-reduction enzymes has been pointed out and the importance of the ratio of nucleic acid to hydrolytic products has been suggested in view of the ready diffusibility of the latter.

Ribonucleic acid and hydrolyzed nucleic acid (mononucleotides) had a negligible effect on cytochrome oxidase; the slight effect in the case of the mononucleotide preparation appeared to be due to the sodium chloride present.

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THE BIOLOGICAL CONVERSION OF *l*-SERINE TO GLYCINE

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The amino acid glycine is involved not only in protein formation but in the synthesis of glutathione, creatine (1), and the protoporphyrin of hemoglobin (2). However, the mechanism by which the animal organism forms glycine is quite obscure. It has been generally considered that the "nonessential" amino acids are synthesized from α-keto acids either by amination with ammonia or by transamination. However, some of the amino acids can be synthesized in the animal cell by transformation of one into another, with utilization of the carbon structure as well as the nitrogen. Evidence has been obtained for the interrelationship of glutamic acid, proline (3, 4), and ornithine (5, 6), of phenylalanine and tyrosine (7), and the conversion of serine into cystine (8, 9).

The observation of Neubauer (10) that phenylglycine gives rise to phenylglyoxylic acids led to the hypothesis that glycine may be formed by a reverse process; *i.e.*, by amination of glyoxylic acid. Interest in this hypothesis has recently been revived by the finding of Ratner, Nocito, and Green (11) that an enzyme exists in liver and kidney which catalyzes the oxidation of glycine to glyoxylic acid and ammonia. However, Haas (12) and Sassa (13) were unable to demonstrate any formation of glycine from this acid in perfusion and feeding experiments.

It has been postulated by Knoop (14, 15) that α-amino-β-hydroxy acids undergo β oxidation to yield glycine, since it had been found that β-phenylserine (14, 16) and δ-phenyl-α-amino-β-hydroxyvaleric acid (15) both yielded hippuric acid when administered to a dog. However, the theory actually sheds no light on the nature of the first 2-carbon fragment that is split off, for benzoic acid should be formed from these odd numbered acids, which in turn would give rise to hippuric acid, regardless of the nature of the 2-carbon fragment.

Recently, Leuthardt (17, 18) found that on incubation of guinea pig liver slices with benzoic acid and glutamine, or *dl*-serine, or asparagine, or *l*-glutamic acid, or proline there was an increase in the α-amino nitrogen in the hydrolyzed ether extract, presumably arising from the hydrolysis of hippuric acid. The most decided increases were produced by glutamine and serine. This finding was positive only in the guinea pig. Leuthardt suggested that both glutamine and serine were converted to glycine in the

liver of the guinea pig. He also found that glutamine was not utilized for hippuric acid formation in the rat liver (17), and neither he (18) nor Borsook and Dubnoff (19) were able to demonstrate the utilization of serine or glutamic acid for hippuric acid formation in the liver slices of the rat.

In order to investigate the origin and mechanism of the formation of glycine *in vivo*, a modification of the isotope dilution technique was employed. Equimolecular quantities (0.35 mM per 100 gm. of body weight) of benzoic acid and of some amino acids labeled with isotopic nitrogen were injected intraperitoneally into fasting rats and guinea pigs. If the labeled compound administered were converted to glycine, the hippuric acid excreted would contain excess N^{15}, for it has been previously demonstrated by Rittenberg and Schoenheimer (20) that the administration of glycine labeled with N^{15}, together with benzoic acid, results in the excretion of labeled hippuric acid. Under these conditions the dietary glycine used for hippuric acid synthesis was diluted by "endogenous" glycine. Since most of the test compounds were labeled only with N^{15}, the appearance of excess isotopic nitrogen in the glycine of the isolated hippuric acid would only be indirect proof for the utilization of the carbon structure of the test compound, for the nitrogen might have been removed from it by deamination and then utilized for glycine formation by combination with some other compound. However, the determination of the ratio of the isotope concentration of the test compound, C_0, to that of the hippuric acid, C, (the dilution factor C_0/C) offers a method of differentiating between direct carbon utilization and mere nitrogen utilization. The dilution factor for glycine should be lower than for any other test compound. This is confirmed by the findings summarized in Table I. The dilution factor for isotopic ammonia was also determined and found to be considerably higher than that of glycine.

The dilution factors of glycine and ammonia can be taken respectively as the lower and upper limits for comparison of "natural" amino acids as glycine precursors. One primary condition that a test compound must meet in order to be considered as a possible precursor of glycine is that its dilution factor must be lower than that found for ammonia. The nearer the dilution factor of the test compound to that found for glycine, the better the evidence for, and the more extensive the conversion.

It can be seen from Table I that, whereas the dilution factors for glycine in the rat and guinea pig are approximately the same, the dilution factors for ammonia in these animals are widely different; *i.e.*, 21 for the guinea pig and 415 for the rat. This indicates that the guinea pig can utilize ammonia more efficiently for the formation of glycine or for a glycine precursor than the rat. Since the dilution factor for ammonia in the rat is so high, the fact that the dilution factors for the other test substances are lower than

that for ammonia is not a sufficient criterion for their conversion to glycine; the test substance may merely be a better nitrogen donor for glycine synthesis than ammonia. On the other hand it may be a relatively poor donor, as appears to be the case for l-leucine in the guinea pig. In the rat it is more desirable to consider how closely the dilution factor of the test substance approaches that of glycine.

It can be seen from Table I that the dilution factor of l-serine more closely approximates that of glycine than any other test compound in both guinea

TABLE I

Utilization of Nitrogen-Containing Compounds for Glycine Formation

0.35 mM of a compound labeled with N^{15} and 0.35 mM of benzoic acid per 100 gm. of body weight were injected intraperitoneally into fasting rats and guinea pigs.

Compound administered		N^{15} excess of isolated hippuric acid (C)		Dilution factor $\left(\frac{C_0}{C}\right)$	
Compound	N^{15} excess (C_0)	Rat	Guinea pig	Rat	Guinea pig
	atom per cent	*atom per cent*	*atom per cent*		
Glycine	1.12	0.406	0.459	2.8	2.4
Ammonia*	2.35	0.006	0.115	390	20
" *	32.5	0.074	1.466	440	22
l-Serine	1.89	0.345	0.483	5.5	3.9
d-Serine	1.89	0.012	0.013	158	145
l-Glutamic acid	0.86	0.019	0.087	45	10
d-Glutamic "	4.50	0.003	0.010	1500	450
dl-Glutamic " †	4.45	0.069	0.345	64	13
dl-Aspartic " †	4.05	0.070	0.218	58	19
l-Alanine	1.97	0.021	0.092	94	21
dl-Proline†	11.6	0.245	0.494	47	23
l-Leucine	4.67	0.039	0.087	120	54
Ethanolamine	2.00	0.006	0.035	334	57

* Administered as ammonium citrate.

† 0.35 mM of the l + 0.35 mM of the d compound per 100 gm. of body weight.

pig and rat, 5.5 in the rat and 3.9 in the guinea pig. This finding strongly indicates an extensive and rapid conversion of l-serine into glycine with utilization of the carbon chain and without loss of the α-amino group, unless a specific transamination is involved. This conversion is specific for the l isomer, as can be seen from Table I. Very little of the labeled N was found in the hippuric acid after the administration of the d-serine. That the metabolic pathways of the two isomers differ is also shown by the work of Artom, Fishman, and Morehead (21) who demonstrated that the d isomer is toxic.

The only other amino acid tested which may be converted into glycine is *l*-glutamic acid (dilution factor 10 for the guinea pig, 45 for the rat). Whether the carbon of glutamic acid is utilized for glycine formation, even in the guinea pig, is difficult to decide, for glutamic acid seems to be the most rapid and effective nitrogen donor for metabolic nitrogen and the value of 10 for the dilution factor in the guinea pig might arise from an efficient transfer of the nitrogen from the glutamic acid to some precursor of glycine. On the other hand, aspartic acid and probably alanine are also efficient nitrogen donors; yet these amino acids are no better than ammonia in the guinea pig. The results here reported, taken in conjunction with those of Leuthardt (17), at least suggest the possibility that glutamic acid may be converted into glycine perhaps by first forming serine by β oxidation. A definite decision must await experiments in which glutamic acid is labeled with heavy carbon.

The presence of a small amount of N^{15} in the hippuric acid when leucine, alanine, aspartic acid, and proline were administered is ascribable to the utilization of the nitrogen of these amino acids, but not of carbon. The apparently more efficient use of the nitrogen of aspartic acid and proline is probably due to the fact that these amino acids were given as a racemic mixture; 0.35 mM of the *l* form and an equal amount of the *d* form were injected. If any of the nitrogen of the *d* isomers was utilized, in contrast to the non-utilization of the nitrogen of the *d*-glutamic acid (Table I) which is largely excreted unchanged (22), the dilution factor would be lower than that which would have been found with the *l* isomer (0.35 mM per 100 gm. of body weight) alone. It would appear therefore that a considerable fraction of glycine is synthesized in the animal body from *l*-serine.

The above data indicate that *l*-serine is directly converted to glycine. The conversion could proceed by any one of three possible mechanisms. (1) The serine could first be decarboxylated to yield ethanolamine, which in turn could be oxidized to yield glycine. (2) The serine could be converted to free aminomalonic acid by oxidation occurring on the β-carbon, which would yield glycine by loss of either carboxyl group. (3) The β-carbon of serine could undergo a partial oxidation to yield formylglycine, which by hydrolytic cleavage would give rise to glycine and formic acid.

In order to elucidate the mechanism of the conversion of serine into glycine and eliminate the remote possibility that only the nitrogen of serine is utilized for glycine formation, serine was synthesized with N^{15} in the amino group and C^{13} in the carboxyl group. This was administered to the rats and guinea pigs, and glycine was isolated from the excreted hippuric acid. The finding of C^{13} in the glycine definitely proves the conversion of serine into glycine and the finding of N^{15} and of C^{13} in almost unchanged ratio in the glycine eliminates ethanolamine as an intermediate (Table II).

This conclusion was corroborated in an experiment in which ethanolamine labeled with N^{15} was administered together with benzoic acid to the animals, and the dilution factor determined after isolating the hippuric acid. It can be seen from Table I that the dilution factor is 57 for the guinea pig and 334 for the rat, showing no conversion of ethanolamine to glycine. The conversion of serine to ethanolamine shown by Stetten (9) may therefore proceed through the intermediate formation of glycine rather than by direct decarboxylation (23).

The approximate equality of the $N^{15}:C^{13}$ ratios contraindicates the participation of free aminomalonic acid as an intermediate, for if this latter compound were first formed, the ratio would be twice as high as that found,

TABLE II

Conversion of dl-Serine, Labeled with C^{13} in Carboxyl Group and with N^{15}, to Glycine

0.35 mM of *dl*-serine (0.175 mM of the d + 0.175 mM of the l compound) and 0.35 mM of benzoic acid per 100 gm. of body weight were injected intraperitoneally into fasting rats and guinea pigs.

Compound injected			N^{15} excess of glycine of isolated hippuric acid (C)		C^{13} excess of carboxyl group of glycine of isolated hippuric acid (C')		N^{15} dilution factor $\left(\dfrac{C_0}{C}\right)$		C^{13} dilution factor $\left(\dfrac{C'_0}{C'}\right)$		$N^{15}:C^{13}$ ratio of glycine of isolated hippuric acid $\left(\dfrac{C}{C'}\right)$	
	N^{15} excess (C_0)	C^{13} excess in carboxyl group (C'_0)	$N^{15}:C^{13}$ ratio $\left(\dfrac{C_0}{C'_0}\right)$	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig	Rat	Guinea pig
	atom per cent	*atom per cent*		*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*						
dl-Serine....	4.53	2.38	1.90	0.614	0.799	0.346	0.410	7.4	5.7	6.9	5.8	1.77	1.95

since either of the two carboxyl groups could be split off. Were aminomalonic acid actually an intermediate, the equality of the $N^{15}:C^{13}$ ratio would be conceivable if serine was reversibly deaminated and by a coincidence the rate of this reaction was of such a magnitude as to leave the $N^{15}:C^{13}$ ratio unchanged. The probability of such a coincidence appears remote; it seems reasonable to conclude that serine is converted into glycine by the splitting off of the β-carbon.

The essential equality of the $N^{15}:C^{13}$ ratios in the serine administered and the glycine isolated could have come about only by the splitting off of the β-carbon in the conversion of serine to glycine and equal utilization of the nitrogen, carboxyl, and α-carbon atoms of serine. The elimination of the β-carbon from serine may take place by a reverse aldolization, as postulated

by Nicolet (24) for the formation of glycine from serine by alkali treatment, or it may take place by β oxidation followed by hydrolytic cleavage, as

$$\underset{\substack{H \\ HO-C-C-COOH \\ H \quad NH_2}}{\overset{H \quad H}{}} \xrightarrow{(-2H)} \left[\underset{\substack{NH_2}}{\overset{H \quad H}{O=C-C-COOH}} \right] H_2O$$

$$CH_2NH_2COOH + HCOOH$$

This would represent an extension of Knoop's theory of β oxidation of fatty acids to a compound containing an amino group on the α-carbon. However, this appears to be a special case in the sense that the β-carbon must first be partially oxidized, for alanine is not oxidized to serine (Table I). Further, both Dakin and Knoop found that while phenylserine (14, 16) and δ-phenyl-α-amino-β-hydroxyvaleric acid (15) gave rise to hippuric acid phenylalanine did not (16). Whether this reaction is general for all α-amino-β-hydroxy acids is still unknown.

The presence of the N^{15} and C^{13} labels of serine in the glycine of the hippuric acid could conceivably be due to the conversion of the l-serine first to N-benzoylserine, and then to hippuric acid. However, this possibility is eliminated by the finding of Magnus-Levy (25) in the rabbit, confirmed by the present author in the rat and guinea pig, that administered N-benzoylserine was excreted unchanged and not converted into hippuric acid.

The observation that the $N^{15}:C^{13}$ ratios in the serine and the glycine are almost equal raises the question of amination of α-keto acids. It would appear that if serine or the glycine formed from the serine is deaminated and resynthesized by amination the over-all process must take place at a very slow rate, for the N^{15} dilution factor would otherwise be much higher than that found for C^{13} and the $N^{15}:C^{13}$ ratio of the isolated glycine much lower. It is well known that the keto acids corresponding to alanine, aspartic acid, and glutamic acid can be aminated by transamination (26, 27) and that α-ketoglutaric acid can be reductively aminated with ammonia to form glutamic acid (28). Also, the finding of N^{15} on the α-amino groups of the essential amino acids, with the exception of lysine (29, 30), and the utilization for growth of some of the d isomers of the essential amino acids (31) show that the α-keto acids of these amino acids can be converted into the corresponding amino acids. Though it is recognized that probably all of the amino acids are deaminated, evidence is accumulating that some of the keto acids are not reaminated either by reductive amination or by transamination. It has recently been shown that arginine does not undergo this reversible reaction (6) and the present report indicates that this is probably true also for serine and glycine.

These findings demonstrate that some of the non-essential amino acids are formed directly from other amino acids rather than by amination of

their keto acids exclusively. Moreover, all relevant information on record suggests that the animal organism can synthesize the carbon chain of only those α-keto acids which arise in the metabolism of the carbohydrates; namely, pyruvic, oxalacetic, and α-ketoglutaric acids. Of the amino acids which arise from these keto acids, glutamic acid may play a central rôle in the formation of other non-essential amino acids. Glutamic acid has been shown to be intimately related to the 5-carbon amino acids proline (3, 4) and ornithine (5, 6). As a result of the present work and that of Leuthardt (17), it appears that glutamic acid may be converted to glycine. The intermediate of this conversion might be serine arising from β oxidation. Moreover, the formation of cystine from serine has been shown (8, 9), and now the conversion of serine into glycine has been definitely established. It is significant that the carbon structure of threonine and the amino acids having more than a 5-carbon chain length, a branched chain, or a ring system do not seem to be synthesized in the rat.

The high degree of utilization of serine (comparison of the dilution factor of glycine and the dilution factor of l-serine) for glycine formation found in these experiments may be misleading, for the animal cell may be carrying out this conversion to an abnormal extent under the stimulus of the necessity for eliminating the benzoic acid. A more significant value should be obtainable by comparing the formation of hemin or creatine from the labeled serine and glycine.

EXPERIMENTAL

Administration of Test Substances—A solution containing 0.35 mM of the neutralized test substance and 0.35 mM of sodium benzoate per 100 gm. of body weight was injected intraperitoneally into the rats (220 to 300 gm.) and guinea pigs (280 to 500 gm.) which were fasted previously for 24 hours. Each test substance was administered to a group of at least two animals. The urine was then collected for 24 hours, during which time food was still withheld from the animals.

Isolation of Hippuric Acid—The urine from the experimental animals of each group was pooled, filtered, acidified to Congo red paper with dilute sulfuric acid, and then extracted with ether for 5 to 8 hours in a continuous extractor. The ether solution was evaporated on a steam bath and the residue recrystallized several times from hot water, charcoal being used when necessary. All the samples of hippuric acid isolated melted at 191–192°.

Glycine from Hippuric Acid—In the experiment in which serine containing C^{13} was administered it became necessary to isolate the glycine from the hippuric acid in order to reduce the dilution of C^{13} by the carbon of the benzoyl group. The hippuric acid isolated from the urine of the rats and

guinea pigs was hydrolyzed by boiling for 5 hours with 20 per cent hydrochloric acid. The solution was diluted with an equal part of water, cooled, and extracted several times with ether. The aqueous solution was taken to dryness *in vacuo* and the residue dissolved in water. The chloride was removed with silver carbonate and the silver removed from the filtrate with hydrogen sulfide. Glycine was isolated from the concentrated filtrate by the addition of alcohol and recrystallized from a solution of water and alcohol.

<p style="text-align:center">$C_2H_5O_2N$. Calculated, N 18.7; found, N 18.8</p>

N^{15} *Glycine*—Glycine was synthesized in the manner described by Schoenheimer and Ratner (32). It contained 1.12 atom per cent excess N^{15}.

N^{15} *Ammonium Citrate*—0.008 equivalent of ammonia generated from isotopic ammonium chloride was distilled into a solution containing 0.02 equivalent of citric acid. One sample contained 2.35, another 32.5 atom per cent excess N^{15}.

N^{15} *l-Glutamic Acid*—The *l*-glutamic acid was prepared by the isolation of *l*-glutamic acid from protein hydrolysates after the addition of isotopic *dl*-glutamic acid to the protein hydrolysate, as described in the isotope dilution method for amino acid analysis (33). It contained 0.862 atom per cent excess N^{15}. The specific rotation, based on free glutamic acid, was $[\alpha]_D = +31.0°$ (2 to 3 per cent in 2.5 N HCl).

N^{15} *dl-Aspartic Acid*—The *dl*-aspartic acid was synthesized in the manner described by Schoenheimer and Ratner (32). It contained 4.05 atom per cent excess N^{15}.

<p style="text-align:center">$C_4H_7O_4N$. Calculated, N 10.5; found, N 10.5</p>

N^{15} *l-Alanine*—*dl*-Alanine containing 1.97 atom per cent excess N^{15} was prepared by condensing acetaldehyde, isotopic ammonium chloride, and sodium cyanide according to the method of Kendall and McKenzie (34). The isotopic *l*-alanine was prepared from the strychnine salt of benzoyl-*dl*-alanine according to the method of Dunn *et al.* (35); $[\alpha]_D = +13.5°$ (3.5 per cent in 6 N HCl).

N^{15} *dl-Proline*—The *dl*-proline was synthesized from isotopic potassium phthalimide (32) by the method of Sörensen and Andersen (36). The proline contained 11.6 atom per cent excess N^{15}.

<p style="text-align:center">$C_5H_9O_2N$. Calculated, N 12.1; found, N 12.1</p>

N^{15} *Ethanolamine, l-Leucine, dl-Glutamic Acid, and d-Glutamic Acid*—The sample of ethanolamine, containing 2.00 atom per cent excess N^{15}, was pre-

pared by Dr. K. Bloch (1), and the samples of *l*-leucine, containing 4.67 atom per cent excess N^{15} (32), *dl*-glutamic acid, containing 4.45 atom per cent excess N^{15}, and *d*-glutamic acid, containing 4.50 atom per cent excess N^{15} (22), were prepared by Dr. S. Ratner. The author wishes to express his thanks for these preparations.

N^{15} *dl-Serine*—Isotopic glycine was prepared as above and esterified with ethyl alcohol. The glycine ethyl ester hydrochloride was benzoylated by the method of Franzen (37). The ethyl hippurate was converted to N-benzoylserine ethyl ester by condensation with ethyl formate and reduction with aluminum amalgam according to the procedure of Erlenmeyer and Stoop (38). Serine was isolated from the above derivative by hydrolyzing with 20 per cent HCl, filtering off the resultant benzoic acid, removing chloride ion with silver carbonate, evaporating, and precipitating with alcohol (7). The serine contained 1.89 atom per cent excess N^{15}.

$C_3H_7O_3N$. Calculated, N 13.3; found, N 13.2

N^{15} *l-Serine and* N^{15} *d-Serine*—The *p*-nitrobenzoyl derivative of the above racemic serine was resolved into the *d* and *l* components by forming the quinine salt of the *d* component and the brucine salt of the *l* component according to the method of Fischer and Jacobs (39).

l-Serine—$[\alpha]_D = -6.8°$ (3.5% in water)
d-Serine—$[\alpha]_D = +7.0°$ (3.5% in water)

N^{15} *and* C^{13} *dl-Serine* ($HO \cdot C \cdot H_2$—$H \cdot C \cdot N^{15}H_2$—$C^{13}OOH$)—Glycine containing C^{13} in the carboxyl group and N^{15} was synthesized by first condensing phthalimide with formaldehyde by the procedure of Sachs (40). The resulting hydroxymethylphthalimide was converted to chloromethylphthalimide by treatment with thionyl chloride and the latter compound was converted to cyanomethylphthalimide by treating it with sodium cyanide, labeled with C^{13}, by the procedure of Sakami, Evans, and Gurin.[1] The cyanomethylphthalimide was hydrolyzed with a mixture of hydrochloric and acetic acids and glycine isolated.[1] The glycine was converted to serine in the manner described above. The serine contained 4.53 atom per cent excess N^{15} and 0.792 atom per cent excess C^{13}; the carboxyl group contained 2.38 atom per cent excess C^{13}.

N-Benzoyl-dl-Serine—N-Benzoyl-*dl*-serine was synthesized by a method similar to that described by Sörensen and Andersen (41); m.p. 169°. 110 and 125 mg. of this compound were injected intraperitoneally respectively into a fasting rat and a fasting guinea pig and the urine of these animals was collected separately for 24 hours. The urine samples were acidified to Congo red paper with dilute sulfuric acid and then extracted with ether for

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[1] Sakami, W., Evans, W. F., and Gurin, S., private communication.

15 hours in a continuous extractor. The ether solutions were evaporated and the residue recrystallized from water with the aid of charcoal. 68 and 76 mg. of N-benzoyl-*dl*-serine were isolated from the guinea pig and rat urines respectively. The isolated material melted at 167–168° and gave no depression in the melting point when mixed with an authentic specimen of N-benzoyl-*dl*-serine.

Isotope Analysis—The N^{15} analysis was carried out in the usual fashion with the mass spectrometer. The C^{13} analysis was carried out by burning the compounds and analyzing the CO_2 with the mass spectrometer. The author wishes to express his appreciation to Mr. I. Sucher for the isotope analyses.

SUMMARY

Amino acids, labeled with N^{15}, together with benzoic acid were injected into rats and guinea pigs and the isolated hippuric acid was analyzed for its N^{15} concentration. By comparing the dilution factor C_0/C ($C_0 = N^{15}$ concentration of administered compound, $C = N^{15}$ concentration of hippuric acid) of several amino acids with that found for glycine and ammonia, it was found that only *l*-serine is directly converted, with utilization of carbon and nitrogen, into glycine in the rat and guinea pig. It would appear from the data that a considerable fraction of *l*-serine is converted to glycine in the rat and guinea pig.

The mechanism of this conversion was established by employing serine labeled with N^{15} in the amino group and with C^{13} in the carboxyl group. It was found that *l*-serine is converted into glycine by the splitting off of the β-carbon atom. It was also found that the $N^{15}:C^{13}$ ratios of the administered serine and of the glycine of the excreted hippuric acid are essentially the same.

The formation of some of the amino acids is discussed.

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THE METABOLISM OF METHYLXANTHINES AND THEIR RELATED METHYLURIC ACIDS*

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In studies on man Myers and Wardell (1) showed that the ingestion of caffeine and theophylline resulted in an increased excretion of uric acid as determined by colorimetric (Benedict-Franke (2), Benedict-Hitchcock (3)) methods, and as. determined in one experiment with theophylline by a gravimetric method. On the other hand no increase in uric acid excretion was noted with the colorimetric method after the ingestion of theobromine. These authors suggested the possibility that the apparent increased excretion of uric acid might be due to partial oxidation of the methylxanthines in position 8 and thus in their conversion to methyluric acids. In support of this hypothesis they found that 1- and 1,3-methyluric acids gave a good color reaction with the Benedict-Franke procedure, that 1,3,7-trimethyluric acid gave a trace of color, while 7- and 3,7-methyluric acids gave no color with the color reagents.

It seemed desirable to study this question further in an effort to elucidate more fully the changes which occur in the methylated xanthines when ingested. Not only are these methylxanthines quite universally consumed by man in such beverages as coffee, tea, and cocoa, but they are also used as diuretics (especially theobromine) and in coronary heart disease (theophylline).

We were fortunate in securing a reasonable quantity of the various methyluric acids from Professor Hermann Biltz of the University of Breslau, and this has permitted us to study the fate of these methyluric acids in the animal body and to compare them with the products excreted in the urine after the ingestion of the methylxanthines.

Preliminary reports of our studies on the methyluric acids and methylxanthines were made some time ago (4,5), but final presentation of the data has been delayed in the hope that we might make microanalyses on the methyluric acids we believed to be excreted after the ingestion of the

methylxanthines. This has not yet been done, but publication of our results should not be delayed longer. Recently Buchanan, with Block and Christman (6, 7), has carried out studies, in which the authors served as subjects, on the fate of the methylxanthines, theophylline, theobromine, and caffeine, with the aid of uricase. Their observations are in complete harmony with our hypothesis and our findings on the influence of the methyluric acids on the apparent uric acid excretion, as determined colorimetrically. These authors state, "The ingestion of caffeine or theophylline by human subjects is followed by a definite increase in the excretion of phosphotungstic acid-reducing materials as measured colorimetrically by both the direct or silver precipitation methods for the determination of uric acid. The administration of theobromine causes no such increase in uric acid excretion. The fractionation of this chromogenic material into true uric acid and residual reducing materials by the use of the enzyme uricase shows that the true uric acid excretion is increased very little, if at all, over a 24 hour period... The ingestion of caffeine or theophylline causes a very definite increase in the excretion of phosphotungstic acid-reducing materials which are not oxidized by incubation with uricase. Evidence has been presented which suggests that these materials may be 1-methyluric acid, 3-methyluric acid, or 1, 3-dimethyluric acid formed by the oxidation and partial demethylation of the methylated xanthines."

Most experiments dealing with uric acid metabolism have been carried out on man, since other mammals, with the exception of man and the anthropoid apes, excrete allantoin as the end-product of purine metabolism rather than uric acid. In 1916 Benedict (8) made the interesting observation that the pure blooded Dalmatian coach-dog excretes uric acid in much the same manner as man. Owing partly to the fact that we desired to study the metabolism of synthetic methyluric acids of unknown toxicity, as well as that of the methylxanthines, the Dalmatian dog was selected as the experimental animal in the crucial part of this study. This was done because we wished to be able to detect any conversion to uric acid in case demethylation occurred in the methyluric acids and demethylation and oxidation in position 8 occurred with the methylxanthines. We realized that in trying to determine the fate of administered methylxanthines and methyluric acids, animals with high uricolytic indexes would possess certain advantages, since uric acid itself would largely be destroyed in the body and thus confusion with other color-reacting substances which might be excreted in the urine would be avoided. Our first studies with the methyluric acids were carried out with the albino rat, which, according to Hunter and Givens (9), has a uricolytic index of 96. However, it is unfortunate that we did not parallel some of our later studies with methyluric acids and methylxanthines made on the Dalmatian dog with similar studies on the common

breed of dog, in which the uricolytic index is 98.   This would have given
us information similar to that obtained by Buchanan *et al.* (7) on human
urine with the aid of uricase.

<center>EXPERIMENTAL</center>

Color Developed with Methyluric Acids—When the different methyluric
acids were dissolved in buffer phosphate solution in the same manner as in
the preparation of the uric acid standard for the Benedict-Franke method
and the color developed, the results given in Table I were obtained.   The
comparison with uric acid is on a gm. molecular basis.   The color develop-
ment was carried out at room temperature, as in the Benedict-Franke
method for urine, but essentially the same results were obtained when the
color was developed in a boiling water bath.   Aqueous solutions of methyl-
uric acids yielded the same amount of color.   Color failed to develop when

<center>TABLE I</center>

<center>*Colorimetric Values for Methyluric Acids with Benedict-Franke Reagents*</center>

Methyluric acid	Color value in terms of uric acid
	per cent
1-Monomethyluric acid...............................	85
1,3-Dimethyluric "	72
3-Monomethyluric "	29
3,9-Dimethyluric "	12
1,3,7-Trimethyluric "	5
7-Monomethyluric "	Mere trace
1,7-Dimethyluric "	" "
3,7-Dimethyluric "	" "

solutions of the methyluric acids were warmed with alkali and carefully
neutralized, and when treated with hydrogen peroxide.

It will be noted in Table I that 1- and 1,3-methyluric acids yield
about three-quarters as much color as uric acid, while 3-methyluric acid
gives less than a third the color and 3,9-methyluric acid gives about an
eighth the color developed by uric acid under similar conditions.   Of the
four methyluric acids with methyl groups in position 7 (7-, 1,7-, 3,7-, and
1,3,7-methyluric acids), only the last responds to the color reaction, and
then only to the extent of 5 per cent.   Buchanan, Block, and Christman
(6), employing samples of the same methyluric acids by a somewhat dif-
ferent procedure for developing the color, obtained results which in general
parallel those of Table I but differ some in percentage values obtained.   For
example, they obtained more color for 1-methyluric acid, less for 1,3-di-
methyluric acid, and about the same for 3-methyluric acid.   With 1,3,7-

trimethyluric acid they report a trace, while we found 5 per cent of color. On the other hand they found 2.2 per cent of color with 1,7-dimethyluric acid, while we obtained only a trace of color.

Solubilities of Methyluric Acids—As a preliminary to devising a scheme whereby it might be possible to isolate the methyluric acids from urine, it seemed necessary to ascertain their solubilities in water. This was determined at 17.5° and 37.5°. With the exception of 3-monomethyluric acid all of the methyluric acids were much more soluble in water than uric acid. 3-Monomethyluric acid is comparatively insoluble, 1 part in about 18,000 parts of water, but the remaining methyluric acids studied were much more soluble, 3,9-dimethyluric acid having a solubility of about 1 in 5000 parts and the remainder being increasingly soluble in the following order, 1-, 3,7-, 1,3-, 1,7-, and 1,3,7-methyluric acids, the last named being soluble in about 200 parts of water. It is evident that on the basis of solubilities it should be possible to separate uric acid from the more soluble methyluric acids by fractional crystallization.

The several methyluric acids are all precipitated by both ammoniacal silver magnesium mixture and Folin's silver lactate-lactic acid solution, but with about half the methyluric acids the precipitation occurs only on standing.

Methods for Metabolism Studies—Total nitrogen was determined by the Kjeldahl distillation and titration method (a micro technique being used with the rat) and creatinine by the original Folin method. The uric acid estimations were made by the Benedict-Franke direct colorimetric method (2) under rigidly controlled conditions. Urines were so diluted that with a standard containing 0.20 mg. of uric acid diluted to 50 cc. after color development and set at 15 mm., the unknowns would read between 10 and 20 mm. in a visual colorimeter. As long as these conditions were strictly followed, the error in the determination of check solutions appeared to be very small.

Experiments with Albino Rat—The animal studies with the methyluric acids were begun with metabolism experiments on the albino rat. In most instances these were of 6 days duration, with a 2 day control period and a 4 day experimental period after intraperitoneal or oral administration of the several methyluric acids. Total nitrogen, creatinine, and uric acid were estimated in the daily urine samples, the constancy in the results for total nitrogen and creatinine being equal to that obtained with the dog. The rat normally excretes very little uric acid (1 to 2 mg. per day). From this point of view the rat should be a favorable animal in which to study the methyluric acids. If the methyluric acids are not acted upon by uricase or otherwise destroyed, they should in all probability be excreted in the urine,

and the methyluric acids which respond to the uric acid color reagent could be recognized and roughly determined in this way.

Nineteen such experiments were carried out. The observations are summarized in Table II. It is at once apparent that only those methyluric acids which give a color response to the uric acid reagent influence the output of substances determined colorimetrically as uric acid. It would

TABLE II

Influence of Administration of Methyluric Acids on Daily Output of Uric Acid As Determined Colorimetrically in Rat

Experiment No.	Methyluric acid administered		How administered	Control averages	Averages of experimental period	Extra uric acid	Recovery
	Type	Amount					
		mg.		*mg.*	*mg.*	*mg.*	*per cent*
1	1-	10	Intraperitoneally	1.6	3.7	8.4	100
2	1-	10	"	2.0	4.2	9.0	
3	1-	20	Orally	2.1	2.8	2.8	14
4	1-	20	"	2.1	2.6	2.0	
5	3-	20	"	0.7	0.7	0	
6	3-	15	Intraperitoneally	0.6	0.5	0	
7	1,3-	23	Orally	1.1	2.4	2.7	20
8	1,3-	17	"	1.2	1.9	3.0	
9	1,3-	9	Intraperitoneally	2.4	3.4	5.0	150
10	1,3-	12	"	1.4	4.3	17.4	
11	3,9-	20	Orally	0.8	0.8	0	
12	3,9-	20	Intraperitoneally	0.8	0.8	0	
13	1,7-	20	Orally	0.9	0.9	0	
14	1,7-	10	Intraperitoneally	1.0	1.1	0	
15	3,7-	20	Orally	0.9	0.9	0	
16	3,7-	20	Intraperitoneally	0.8	0.9	0	
17	1,3,7-	22	Orally	0.9	0.9	0	
18	1,3,7-	10	Intraperitoneally	1.0	1.3	1.3	300
19	1,3,7-	10	"	1.1	1.5	1.7	

Controls are averages of 2 to 4 days, and experimental periods averages of 2 to 6 days (4 days in sixteen experiments). The recovery is calculated on the basis of the colorimetric values for the methyluric acids given in Table I.

appear that 1- and 1,3-methyluric acids are quite completely excreted in the urine when given intraperitoneally, but that fully 80 per cent disappears when administered orally. In the case of 1,3,7-trimethyluric acid the recovery is 3 times the theoretical without some demethylation of this methyluric acid. Since this methyluric acid yields only 5 per cent the color of uric acid, the figures for extra uric acid were multiplied by 20. The chance for error here is large, but it seems probable that there has been some

demethylation in position 7, resulting in increased color development. The observations on 1-, 1,3-, and 1,3,7-methyluric acid made on the rat harmonize very well with the data given in Table I, although 3- and 3,9-methyluric acids, which yield some color with the uric acid reagent, did not influence the apparent uric acid excretion after ingestion. It may be noted that of the seven different methyluric acids studied these two are much the least soluble. However, a more probable explanation for the absence of any extra uric acid would be the demethylation of the methyluric acids, especially 3-methyluric acid, and subsequent destruction of the uric acid, since an increased excretion of uric acid was found in similar experiments on the Dalmatian dog, as shown below.

Experiments with Dalmatian Dog—The experiments were carried out on a Dalmatian bitch of known pedigree, purchased from kennels specializing in this breed. At the beginning of the experiment it was 10 months old and weighed 15.5 kilos, a weight maintained throughout the experiments. The diet was purine-free, but, since there were some variations in the control uric acid values in the several periods in which the methyluric acids and methylxanthines were administered, the control data for total nitrogen, creatinine, and uric acid are given in Table III to aid in the evaluation of the colorimetric uric acid outputs for the experimental periods. The 24 hour urine specimens were separated at the same hour daily by catheterization.

Normal Uric Acid Excretion—From the beginning of the experiments in March, until June 6, 1929, the dog was fed on a diet of bread and milk plus cod liver oil and salt mixture (10). It was calculated to contain 1870 calories. Benedict had maintained a Dalmatian dog on such a diet for a period of a year without any deleterious results. During the summer the dog was kept on a farm, the diet being kitchen scraps and milk. In the fall, when brought back to the laboratory, the bread and milk diet was resumed. This resulted after a few days in an increased output of both total nitrogen and uric acid, as will be noted in Table III, the uric acid rising from a 24 hour average of 416 mg. to 582 mg. On this account the diet was reduced to 1400 calories on September 23. Although a positive nitrogen balance was maintained, the uric acid in the control period, October 11 to 13, fell to a daily average of 325 mg. From then on until the end of December the endogenous uric acid output gradually rose, and from December 21 to 23 averaged 427 mg. In January the synthetic diet of Cowgill (10) containing 1243 calories was used, and there was again a considerable drop in the endogenous uric acid output, the average of the three final periods being 307 mg.

The increased output of uric acid which occurred on resumption of the bread and milk diet in September and the subsequent drop which occurred

in October, when the diet was reduced 25 per cent, were obviously due to changes in the diet. Later in November and December, while on this same reduced bread and milk diet, the uric acid returned nearly to its initial level. It again dropped in January when the Cowgill diet with a 10 per cent further reduction in calories was employed. It will be noted that the changes which occurred in the uric acid output were far greater than those in total nitrogen. There is no reason to suppose that the ingestion of the methyluric acids or methylxanthines had more than a temporary influence

TABLE III

Control Daily Total Nitrogen, Creatinine, and Uric Acid Excretion in Dalmatian Dog
(Daily Averages for Periods Given)

Time, 1929-30	Total nitrogen	Creatinine	Uric acid
	gm.	*mg.*	*mg.*
Mar. 11–13	4.99	424	449
" 20–22	5.00	417	443
" 29–Apr. 1	5.41	424	483
Apr. 12–14	5.81	426	461
" 17–19	5.31	434	459
" 23–25	6.65	476	438
" 28–30	6.05	515	458
May 16–18	5.71	506	478
" 30–June 1	6.36	518	462
Sept. 10–11	7.91	550	416
" 12–16	8.91	527	546
" 17–22	8.85	537	582
Oct. 11–12	7.33	511	325
" 20–22	7.09	527	368
" 29–31	7.57	522	326
Nov. 8–10	7.52	503	415
" 25–27	7.81	502	422
Dec. 4–6	8.08	492	432
" 21–23	8.01	501	427
Jan. 1–5	7.69	527	305
" 11–13	7.75	503	297
" 19–21	6.91	520	319

on the colorimetrically determined uric acid and therefore would not have been a factor in the different uric acid levels found in the several control periods. Evidently there are factors governing the endogenous formation and excretion of uric acid which are not fully understood despite the large amount of study this subject has received in the past. Further studies on the Dalmatian dog might help to elucidate some of them, since the endogenous output of uric acid in this animal is considerably larger than in man. It is interesting to note that in comparison with a man weighing 70 kilos

the output of endogenous uric acid in this Dalmatian dog was about 6 times as great, while the total nitrogen was greater by about 3 times and the creatinine by 1.5 times. It will be noted that the values for creatinine rose during the first 6 weeks, possibly because the dog had not reached full maturity, but during the following 9 months remained very constant.

Administration of Methyluric Acids—Seven different methyluric acids were administered to the Dalmatian dog by mouth, and the output of colorimetrically determined uric acid compared with the control output observed for the 3 days preceding the administration of the methyluric acid. Generally the output of uric acid was estimated for 5 days following the

TABLE IV

Influence of Ingestion of Methyluric Acids on Excretion of Uric Acid As Determined Colorimetrically in Dalmatian Dog

		Uric acid excretion			
Methyluric acid studied	Amount of methyluric acid ingested	3 day averages	After methyluric acid, 5 day averages	Extra uric acid	Recovery in urine
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
1,3-	500	462	549	+435	125
1,3-	600	443	535 (6)	+552	
1-	400	449	495	+230	75
1-	400	478	535	+285	
3-	300	458	492	+170	150
3-	450	422 ●	448 (6)	+150	
1,3,7-	445	438	448 (4)	+40	180
3,9-	400	459	446	+35	75
3,7-	400	461	458	−15	0
1,7-	400	483	473	−50	0

Controls are averages of 3 days, and the experimental period averages of 5 days, except as noted. The recovery is calculated on the basis of the colorimetric values for the methyluric acids given in Table I.

The numbers in parentheses give the number of days used to compute the averages when these are more or less than 5.

administration of the methyluric acid. The difference between the average control output of uric acid and that for the experimental period was multiplied by the length of the experimental period in days, and the total difference taken to indicate the output of the phosphotungstic acid-reducing material due to the ingestion of the methyluric acids in terms of uric acid. The recovery of the methyluric acids ingested is calculated on the basis of the color values for the different methyluric acids given in Table I. The data are presented in Table IV. It is apparent that 1,3-, 1-, and 3-methyluric acids are recovered quite completely in the urine after oral administration to the Dalmatian dog. The results are probably more accurate

for 1,3- and 1-methyluric acids than for 3-methyluric acid, since the phosphotungstic acid-reducing values of the first two methyluric acids are much more nearly equal to uric acid than is the case with 3-monomethyluric acid. It is of interest that the apparent recovery of 1,3-dimethyluric acid was somewhat better than for 1-monomethyluric acid in both the rat and the dog. There was no apparent recovery of 3-monomethyluric acid in the rat, while recovery in the dog was apparently quite good. These observations can be reconciled on the basis of considerable demethylation of 3-monomethyluric acid. It has long been recognized that the methyl group in position 3 is the least stable (11, 12). If demethylation occurred in the rat, there would be conversion to uric acid and this would be destroyed. If there was partial conversion to uric acid in the Dalmatian dog, the recovery should be greater than the theoretical, since 3-monomethyluric acid yields only about a third as much color as uric acid. The recovery on the basis of the color reaction was about 150 per cent. Similarly the rat showed no increase after the administration of 3,9-dimethyluric acid, while there appeared to be some increase in the dog. The data on 1,3,7-trimethyluric acid suggest quite strongly not only that this substance is excreted in the urine, but that there has been appreciable removal of the methyl group in position 7, despite the fact that this methyl group has been regarded as the most stable one (11) and least inclined to electrolytic dissociation (12). As with the rat, the experiments with 3,7- and 1,7-dimethyluric acids were negative. This is to be expected, since neither reacts with the uric acid color reagent. Furthermore, the negative findings indicate that in these two dimethyluric acids appreciable demethylation did not occur in position 7.

Administration of Methylxanthines—Data which summarize our observation on the administration of the methylxanthines to the Dalmatian dog are given in Table V. Unfortunately it was during the period of these experiments that we encountered the greatest difficulty in holding the level of the endogenous uric acid constant. The dates are given so that the experiments may be correlated with data given in Table III. However, it may be noted that in each experiment the uric acid returned to the approximate level of the control value after the methylxanthines given had been excreted. There is unmistakable evidence of the increased excretion of phosphotungstic acid-reducing material after the administration of theophylline. If these values are recalculated to 1,3-dimethyluric acid on the basis of the color-yielding value of this methyluric acid, the recovery almost exactly equals 100 per cent. We know from the experiments reported on the rat and the dog that 1,3-dimethyluric acid appears to be quite completely excreted in the urine. From the experiments recorded in Table V it would appear that theophylline is almost quantitatively oxidized in

position 8 and thus converted to 1,3-dimethyluric acid, and that this methyluric acid is then excreted in the urine. As discussed below, it has been possible to isolate crystals from the urine, after theophylline administration, which appear identical with 1,3-dimethyluric acid.

The increase in the excretion of phosphotungstic acid-reducing material after the ingestion of caffeine is unmistakable. It is unfortunate that the control values for the endogenous uric acid were at a low level, but there was a marked rise in the uric acid output, up to 250 mg., after administration of the caffeine given in divided doses on 2 successive days, with subsequent return to the control level. If one calculates the extra phosphotungstic acid-reducing material as 1,3,7-trimethyluric acid, which yields

TABLE V

Influence of Ingestion of Methylxanthines on Excretion of Uric Acid As Determined Colorimetrically in Dalmatian Dog

Date of experiment, 1929–30	Methylxanthine studied	Amount of methylxanthine ingested	Uric acid excretion		
			Control 3 day average	Methylxanthine administration, 6–10 day average	Extra uric acid, 6–10 days
		mg.	mg.	mg.	mg.
Jan. 19–30	Theophylline	2000	319	525 (9)	+1854
Nov. 8–20	"	1500	415	546 (10)	+1310
Oct. 29–31	"	750	326	429 (7)	+721
Dec. 21–31	"	750	427	497 (8)	+560
Jan. 11–21	Caffeine	1250	297	410 (8)	+904
Oct. 11–20	"	1000	325	431 (7)	+742
Jan. 1–12	Theobromine	2600	305 (5)	347 (7)	+294
Oct. 20–28	"	1250	368	344 (6)	−198

The numbers in parentheses give the number of days used to compute the averages.

only 5 per cent the color of uric acid, then the recovery is about 15 times that calculated. This would suggest strongly that there has been considerable demethylation in position 7 resulting in the formation of methyluric acids which yield much more color than 1,3,7-trimethyluric acid.

Two experiments were carried out with theobromine. In one, 750 and 500 mg. of the methylxanthine were administered 3 days apart with little change in the output of uric acid. In the second experiment 600, 1000, and 1000 mg. of theobromine were administered on successive days. On the days with the two large doses there appeared to be a definite rise in the output of uric acid, but this returned to the control level on the succeeding day. However, if one compares these experiments with those in which theophylline and caffeine were administered, the change in uric acid output was slight.

Isolation of 1,3-Dimethyluric Acid after Administration of Theophylline—
Isolation of crystals which appeared identical with 1,3-dimethyluric acid
after the administration of theophylline to the Dalmatian dog was secured
by the following procedure. An amount of urine containing approximately
200 mg. of phosphotungstic acid-reducing substances in terms of uric acid
was employed. The urine was precipitated with ammoniacal silver mag-
nesium mixture, the precipitate allowed to settle in a dark cabinet for 30
minutes, then thrown down in a centrifuge. The precipitate was transferred
to a beaker with water and decomposed with hydrogen sulfide, the excess
hydrogen sulfide expelled, the material filtered while hot, and then taken to
dryness on a water bath. The impure uric acid mixture was dissolved in
boiling water and, while still warm, reprecipitated with ammoniacal silver
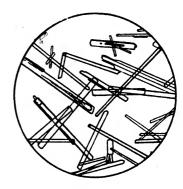
FIG. 1.   FIG. 2.

FIG. 1. Crystals obtained from urine following theophylline administration.
FIG. 2. Crystals of 1,3-dimethyluric acid.

magnesium mixture, the original process being carried to the final stage of
concentration. When the volume had been reduced to about 400 cc., it
was strongly acidified with HCl and the solution set aside in a cool place
for the uric acid to crystallize out. After standing for 24 hours, a mass of
crystals had separated. These showed the quite typical microscopic ap-
pearance of uric acid and were filtered off. The mother liquor was then
concentrated to 100 cc. and again acidified with HCl. The crystals
which formed were more typical of 1,3-dimethyluric acid than of uric acid,
but they were separated, removed, and the mother liquor concentrated to
25 cc., and again allowed to crystallize. The crystals which formed were
separated and then recrystallized. The amount of material obtained was
quite small, but the crystals were typical of 1,3-dimethyluric acid. The
crystals obtained are shown in Fig. 1, in comparison with crystals of pure

synthetic 1,3-dimethyluric acid shown in Fig. 2.   The drawings are accurate copies of photomicrographs.[1]

DISCUSSION

The older literature on the fate of the methylxanthines in the body has been discussed in a previous paper (1), and references will be made here only to work that has a direct bearing on our present studies.   Until the advent of colorimetric methods of uric acid estimation it was not believed that the ingestion of the methylxanthines (caffeine, theobromine, and theophylline) influenced the excretion of uric acid in the urine.   It was thought that the change which occurred in the methylxanthines in the body was simply partial demethylation.   From 10,000 liters of normal human urine Krüger and Salomon (13) were able to isolate about 95 gm. of purines, of which 31 gm. were 1-methylxanthine, 22 gm. were 7-methylxanthine, and 15 gm. were 1,7-dimethylxanthine.   Later Krüger and Schmid (11) concluded that there was good evidence in man that the methyl groups are decreasingly stable in the order 7,1,3, and on this basis the methylxanthines found in human urine can be easily accounted for by the removal from the methylxanthines ingested of the methyl group occupying position 3.

After the introduction of colorimetric methods of uric acid estimation, an increase in the output of phosphotungstic acid-reducing material was observed following the administration of caffeine by Benedict (8), and after coffee and tea and decaffeinated coffee plus caffeine by Mendel and Wardell (14).   Subsequently Myers and Wardell (1) observed that, although they obtained an apparent increase in the excretion of uric acid as determined by colorimetric method after caffeine and theophylline, there was no such increase after the ingestion of theobromine.   Clark and de Lorimier (15) also studied the influence of caffeine and theobromine on the excretion of uric acid with a colorimetric method and likewise observed that theobromine was without influence on the uric acid output.   The possibility occurred to Myers and Wardell that this discrepancy might be explained by the oxidation of the methylxanthines in position 8 to methyluric acids and that these might vary in their response to the colorimetric procedure of estimating uric acid.   Colorimetric tests carried out on methyluric acids kindly furnished by Professor Biltz were found to support this hypothesis.   In their recent report Buchanan, Christman, and Block (7) have shown conclusively that, whereas there is a marked increase in the excretion of phosphotungstic acid-reducing material after both theophylline and caffeine, there is no increase in the excretion of true uric acid, as de-

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[1] Figs. 1 and 2 are drawings of photomicrographs made by Theodora Bergsland of the Institute of Pathology, Western Reserve University.

termined with the aid of uricase. Theobromine was without influence on the excretion of phosphotungstic acid-reducing material. Their studies would appear to have finally disproved the view that there is any molecularly complete demethylation of the methylxanthines resulting in their conversion to uric acid.

We believe that our studies with the methyluric acids and methylxanthines have brought measurably nearer the final answer to the fate of the methylxanthines. Certainly the conclusion that the methylxanthines are at least partially oxidized in position 8 to form methyluric acids now seems inescapable. Our observations appear to support the conclusion of Krüger and Schmid (11) and Biltz and Hermann (12) that the methyl group in position 3 is the least stable in the methylxanthines (also in the methyluric acids). The methyl group in position 7 has been regarded as the most stable, but our observations on caffeine and 1,3,7-trimethyluric acid strongly suggest that there must be some demethylation in this position; otherwise it would be impossible to account for the increase in excretion of phosphotungstic acid-reducing substances observed after their administration.

Observations which support the above statements will be summarized briefly. When 3-methyluric acid is given to the rat, which has a uricolytic index of 96, no extra uric acid is excreted, but in the Dalmatian dog the extra uric acid considerably more than accounts for the 3-methyluric acid given. If the methyl group in position 3 is removed, uric acid will be formed. In the rat it would be destroyed but in the Dalmatian dog it would increase the amount of phosphotungstic acid-reducing material, since uric acid yields about 3 times as much color as 3-methyluric acid.

It would not appear that significant demethylation occurred in position 1 when 1-methyluric acid was administered. This methyluric acid yields 85 per cent of the color given by uric acid. When administered intraperitoneally to the rat, there was 100 per cent recovery. If any uric acid had been formed it would have been destroyed. The apparent recovery in the urine of the Dalmatian dog was about 75 per cent.

When 1,3-dimethyluric acid was administered intraperitoneally to the rat and orally to the Dalmatian dog, there were 150 and 125 per cent recovery, respectively. Since 1-methyluric acid yields about 13 per cent more color than 1,3-dimethyluric acid, the increase in phosphotungstic acid-reducing material can be accounted for by some demethylation in position 3. In the four experiments with theophylline the average recovery of extra uric acid accounts for about 100 per cent conversion to 1,3-dimethyluric acid, as determined colorimetrically. Certainly one must conclude that fairly complete oxidation has occurred in position 8. While it seems likely on the basis of the experiments with 1,3-dimethyluric acid

that some demethylation occurred in position 3, this conclusion cannot be drawn from the data available.

According to Biltz and Hermann (12) the inclination of the methyl groups towards electrolytic dissociation is in the order of positions 3,9,1, and 7. When 3,9-dimethyluric acid was administered to the rat, no extra reducing material was detected, but with the Dalmatian dog there was fairly good recovery. While our experiments are insufficient to draw a final conclusion, they do suggest demethylation in both positions 3 and 9.

When 1,3,7-trimethyluric acid was administered intraperitoneally to the rat, the recovery in the urine was calculated as 300 per cent. Oral administration to the Dalmatian dog gave a recovery of 180 per cent. The error in the calculation is obviously large, since 1,3,7-trimethyluric acid yields only 5 per cent of the color given by uric acid; nevertheless the findings strongly suggest that there has been some demethylation in position 7, thus yielding a methyluric acid with a higher chromogenic value. Demethylation in position 1 or 3 would have decreased, not increased, the chromogenic value with phosphotungstic acid. In the two experiments in which caffeine was administered to the Dalmatian dog, the recovery of extra uric acid is about 15 times that calculated for 1,3,7-trimethyluric acid. This would suggest that the caffeine was not only quite completely oxidized in position 8, but that a considerable part of the methyl group in position 7 must have been removed. It is of interest to note that, in relation to the phosphotungstic acid-reducing power of 1,3,7-trimethyluric acid, uric acid yields 20 times as much color, 1-methyluric acid 17 times as much color, and 1,3-dimethyluric acid 15 times as much color. This last relationship is the one which was actually observed in the experiments with caffeine.

SUMMARY

The metabolism of 1-, 3-, 1,3-, 1,7-, 3,7-, 3,9-, and 1,3,7-methyluric acids has been studied in the albino rat, an animal with a high uricolytic index, and in the Dalmatian dog, an animal which resembles man in its excretion of uric acid. The metabolism of theophylline, theobromine, and caffeine was also studied in this dog.

The most significant findings regarding the methyluric acids are that 3-methyluric acid appears to be quite completely demethylated and converted to uric acid, that 1,3-dimethyluric acid is eliminated in considerable part unchanged, although there may be some demethylation in position 3, and that 1,3,7-trimethyluric acid is partially demethylated in position 7.

The methylxanthines, theophylline and caffeine, appear to be quite completely converted to methyluric acids by oxidation in position 8. This question cannot be answered regarding theobromine with our present methods, since 3,7-dimethyluric acid is not reduced with phosphotungstic

acid. It would appear that the larger part of theophylline was eliminated as 1,3-dimethyluric acid, although some may be excreted as 1-methyluric acid. In the case of caffeine the increased excretion of phosphotungstic acid-reducing material can be explained most simply by oxidation in position 8 and fairly complete demethylation in position 7.

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MICROMETHOD FOR THE DETERMINATION OF UREA

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The need for a micromethod to determine urea in micro samples of plasma, aqueous humor, and other body fluids has been met by the simple procedure to be described. The test can be used to determine the urea content of plasma or aqueous humor in quantities as small as 4 c.mm. with an accuracy of ±5 per cent. It will be apparent that other nitrogen-containing compounds similarly may be determined by this method; the minimal size of samples should contain about 1 γ of nitrogen. The method is essentially a combination of that of Conway (1) and Prater, Cowles, and Straka (2), appropriately modified for micro quantities. It consists first in conversion of urea to ammonia, secondly, in distillation into a drop of glycerol containing boric acid, and third, in titration of the distillate with dilute acid. The procedure is well adapted to making multiple determinations.

Apparatus—Two means were investigated to accomplish the distillation. To keep the time to a minimum, the distance separating the surface of the sample to be analyzed from the drop into which the ammonia is to be distilled must be small. The simplest method of accomplishing this consisted in surrounding small glass vials (9 mm. inside diameter and 6 mm. deep) with wells approximately 1 mm. in depth reamed out of a $\frac{1}{2}$ inch Leucite plate, the diameters being of a size which easily accommodated the sides of a second glass vial (16 mm. outside diameter and 1 cm. deep) inverted over the first vial. The inner and outer walls were 14 and 18 mm., respectively. A top and side view of the apparatus is shown in Fig. 1, *A*.

In the second method a well of the same diameter, but deeper (8 mm.), was reamed out of the Leucite plate, and a hole having a diameter of 8 mm. and a depth of 4 mm. was drilled in the center, as shown in Fig. 1, *B*. With either method the distance between the surface of the liquid and the drop was less than 0.5 cm. None of the dimensions appeared to be critical. The only advantage of the second method is the elimination of one set of vials and the consequent ease of cleaning and lack of breakage.

Any micro pipette or micro burette can be used to measure the sample. We used a micro pipette containing 0.05 ml. graduated in 0.0005 ml. The micro pipette[1] has a ground male joint on one end to receive a 27 gage

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[1] Available from the Macalaster Bicknell Company, Cambridge, Massachusetts.

hypodermic needle and a ground female joint on the other end to hold a tuberculin syringe. The use of this type of micro pipette made it possible to obtain samples of body fluids such as aqueous humor, and, without transferring the material, to measure the samples accurately. A micro pipette like the one described above, but designed to hold 0.15 ml., was used for the titration.

Stirring was most easily accomplished by introducing "fleas" consisting of a small piece of iron wire coated with glass in each vial, and placing the vial in a rotating magnetic field,[2] although satisfactory results were obtained when stirring was done manually.
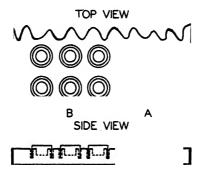
FIG. 1. Apparatus used for determination of urea. A, wells 1 mm. deep; B, wells 4 mm. deep, 8 mm. in diameter.

Procedure

Reagents—

Glycerol-boric acid. To 100 ml. of 25 per cent glycerol, saturated with boric acid, are added 25 ml. of pure glycerol. (This prevents the boric acid from precipitating during the distillation process.)

Urease extract (1 gm. of Squibb's powdered double strength urease) in 100 ml. of boiled saturated sodium chloride.

Saturated sodium metaborate in saturated potassium chloride (boiled).

0.002 N hydrochloric acid.

Gramercy universal indicator (Fisher Scientific Company) diluted with 15 parts of distilled H_2O.

Mineral oil.

The sample to be analyzed is measured either into the small vial or a central cup (Fig. 1), and 1 drop of urease solution is added and allowed to react for 20 minutes at room temperature. Several drops of mineral oil are put in the well, and a small drop of the glycerol-boric acid solution is

[1] Magnetic stirrer, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

placed in the center of the larger vial. The size of the drops should be approximately the same. We used 0.005 ml. and measured it with a micro pipette. After the incubation period I drop of the metaborate solution is added and the vial containing the glycerol drop is immediately inverted over the central cup; the glycerol will remain as a hanging drop. 60 minutes at 30° are allowed for distillation. The vial containing the distillate is then blotted to remove adhering oil, 0.5 ml. of the diluted indicator is added, and the solution is titrated with 0.002 N HCl to the color of a control containing no urea. The latter should be yellow. A lapse of approximately 1 minute should be allowed between the addition of the final content of acid and the attempt to match the end-point. If the control has a greenish appearance, it indicates that some ammonia has been introduced, probably from the NaCl used to make up the urease or from the KCl-metaborate solution. It is recommended that several standards containing known amounts of urea be run with each set of unknowns.

A colorimetric procedure described by Russell (3) may be substituted in place of the titration, if desired. In this instance the glycerol drop is washed out of the vial with three 0.5 ml. portions of water into colorimeter tubes. To each tube is added 0.05 ml. of 0.003 M Mn_2SO_4 or $MnCl$. With the tubes well chilled, 1.0 ml. of alkaline phenol reagent (25 per cent phenol in 2.7 N NaOH) and 0.5 ml. of hypochlorite solution are added. The tubes are then placed in a boiling water bath for 5 minutes, cooled, diluted to convenient volume, and read in a colorimeter.

Results

The time required for completion of the distillation process was determined first. Urea solutions containing 3.6 γ of ammonia nitrogen were incubated with urease and permitted to distil for various lengths of time before titration. The results of analyses performed in triplicate are averaged and appear in Fig. 2. It will be seen that maximal values were obtained after 45 minutes distillation. On the basis of these experiments a distillation time of 60 minutes was used, and found to give satisfactory results.

The method was then tested by making analyses of known amounts of urea. A typical series of results is given in Table I which shows the quantity of urea recovered and per cent of theoretical.

The results obtained through the use of the colorimetric method which can be substituted for the titration procedure are illustrated in Fig. 3. The sensitivity of this method approximates that of the titration procedure.

The efficiency with which known amounts of urea could be recovered from plasma was then determined. Urea in amounts varying from an equivalent of 2 to 4 γ of ammonia nitrogen was added to 0.01 or 0.02 ml. of
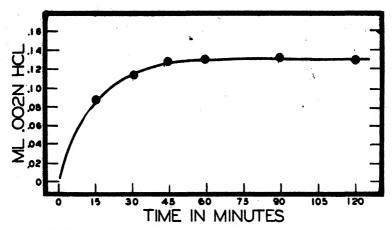
FIG. 2. Distillation of ammonia into glycerol-boric acid solution at varying time intervals.

TABLE I

Urea Determinations

Ammonia nitrogen		Theoretical
Added	Found	
γ	γ	per cent
1.00	1.00	100
1.00	0.98	98
1.00	1.00	100
1.00	0.91	91
1.00	0.96	96
1.00	1.01	101
2.00	2.10	105
2.00	2.02	101
2.00	2.05	102
2.00	2.04	102
2.00	2.02	101
2.00	2.02	101
3.00	3.13	104
3.00	3.04	101
3.00	3.02	100
3.00	2.99	100
3.00	2.96	99
3.00	2.99	100

rabbit plasma of known urea content. The mixture was then analyzed for urea in the usual manner and the findings are presented in Table II. It may be seen that an average of 97.8 per cent (range 94 to 101 per cent) of the

quantity of urea added to plasma was recovered.   A similar proportion of
urea added to aqueous humor was also recovered.
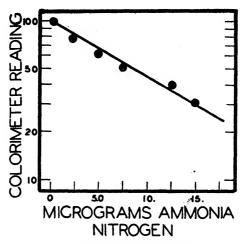
FIG. 3. Analyses of standard urea solutions

TABLE II

Recovery of Urea from Rabbit Plasma

Urea		Total urea		Difference	Urea recovered
Present	Added	Theoretical	Found		
γ	γ	γ	γ	γ	*per cent*
2.91	4.23	7.14	6.90	−0.24	96.8
3.00	8.35	11.4	10.9	−0.50	96.0
3.18	4.23	7.41	7.30	−0.11	98.5
3.18	8.47	11.7	11.1	−0.60	95.0
4.50	7.80	12.3	12.1	−0.20	99.0
4.50	7.80	12.3	11.6	−0.70	94.0
5.85	2.54	8.39	8.25	−0.14	98.4
5.85	4.23	10.1	9.75	−0.35	96.5
5.85	5.94	11.8	11.6	−0.20	98.0
5.85	8.47	14.3	13.5	−0.80	94.4
6.00	8.35	14.4	14.5	+0.10	101.0
Average...					97.8

Since the chief purpose of developing this micromethod was to make
replicate quantitative determinations of urea in body fluids, particularly
aqueous humor, a series of analyses was made of the urea content of serum
and aqueous humor from young adult albino rabbits, and the ratio of the

quantity of urea present in the aqueous humor to that in the plasma was calculated on the basis of their respective water contents. In all instances, duplicate analyses were made on 0.013, 0.026, and 0.039 ml. of plasma or aqueous humor (obtained from one eye in each instance), and the quantities of urea present were estimated from the best line connecting these points. The findings are shown in Table III, whence it may be seen that, with one exception, the quantity of urea present in the plasma varied between 236 and 347 γ per ml. of water. The ratio of urea in the aqueous humor to that in the plasma varied between 0.73 and 0.97, the average being 0.88.

TABLE III

Urea Concentration Present in Plasma and Aqueous Humor of Rabbits, and Ratio of Their Concentrations

Rabbit plasma	Aqueous humor	Ratio, aqueous to plasma
γ per ml. water	*γ per ml. water*	
255	216	0.85
276	248	0.90
289	215	0.74
255	187	0.73
278	213	0.77
324	303	0.935
665	590	0.89
308	287	0.93
268	238	0.89
347	337	0.97
247	230	0.93
300	270	0.90
236	217	0.92
238	225	0.945
Average..306	270	0.88

The latter value is considerably higher than the ratio of 0.65 found previously by Kinsey and Grant (4) with an aeration method which required samples of aqueous humor pooled from two eyes, for a single determination. The significance of this finding will be discussed elsewhere.

SUMMARY

A direct micromethod of estimating urea in plasma or other body fluids in quantities as small as 4 c.mm. containing 1 γ of urea with an accuracy of ± 5 per cent is described. The method consists in direct conversion of urea into ammonia, distillation into a hanging drop of glycerol containing boric acid, and titration with dilute acid, or, alternatively, with a colorimetric procedure in place of the acid titration.

Average recoveries of urea from 0.01 to 0.02 ml. of plasma or aqueous humor were 97.8 per cent with a variation of 94 to 101 per cent.

The quantity of urea in rabbit plasma and aqueous humor averaged 306 and 270 γ per ml. of water, respectively, with an average ratio of 0.88.

The authors wish to acknowledge the technical assistance of Miss Sylvia Landy.

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THE SYNTHESIS AND PROPERTIES OF THE ACYL PHOSPHATES OF SOME HIGHER FATTY ACIDS

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In preceding communications from this laboratory (1, 2) on the activation of fatty acid oxidation by adenosine triphosphate, a working hypothesis on the nature of this activation was elaborated. This hypothesis suggested the possible intermediate formation of carboxyl phosphates of the fatty acids to account for the necessity of adenosine triphosphate in fatty acid oxidation. The subject of this paper is the synthetic preparation of the higher fatty acid phosphates, which will allow an experimental trial of these compounds as intermediates both in fatty acid oxidation, as mentioned above, and also in connection with Lipmann's suggestion (3) that they may be intermediates in the synthesis of the glyceride bond according to the equation

$$\text{(1)} \quad \underset{\text{O}}{\overset{\|}{\text{RC}}}-\text{OPO}_3\text{H}_2 + \text{HO}-\overset{|}{\underset{|}{\text{C}}}- \longrightarrow \underset{\text{O}}{\overset{\|}{\text{RC}}}-\text{O}-\overset{|}{\underset{|}{\text{C}}}- + \text{H}_3\text{PO}_4$$

Fatty acid phosphates are the mixed anhydrides of phosphoric acid and a fatty acid. As such they show instability in aqueous media: the 1,3-diphosphoglyceric acid isolated by Negelein and Brömel (4) as a product of glyceraldehyde phosphate oxidation has a half life in neutral aqueous solution at 37° of some 27 minutes; the acetyl phosphate synthesized by Lynen (5), approximately 187 minutes under the same conditions. Furthermore, the phosphate of these compounds is very labile in the presence of the molybdate-containing reagents, so that it appears as inorganic phosphate during the usual phosphorus partition procedures. These properties illustrate the difficulties involved in the preparation, purification, and detection of such compounds.

Lynen (5) first described the synthesis of monoacetyl phosphate. This was prepared by the reaction of silver dibenzyl phosphate with acetyl chloride, followed by removal of the benzyl groups as toluene by catalytic hydrogenolysis. Lipmann and Tuttle (6) recently published a much more convenient synthesis of acetyl phosphate, based on the reaction between

acetyl chloride and monosilver phosphate (prepared by reaction of 2 moles of H_3PO_4 and 1 of Ag_3PO_4). The use of silver dibenzyl phosphate and monosilver phosphate is necessary to prevent the formation of di- and triacetyl phosphates, which are formed when trisilver phosphate is used and which, according to Lynen (5), are impossible to convert to the monoacetyl compound by partial hydrolysis.

The syntheses of pure monooctanoyl and monopalmityl phosphate described in this report were completed independently before Lipmann and Tuttle's detailed report on acetyl phosphate appeared. They were based, however, on an earlier statement by Lipmann (7) that a mixture of phosphoric acid and trisilver phosphate reacted with acetyl chloride to form monoacetyl phosphate, indicating that monosilver phosphate, which has never been described, was formed by the action of H_3PO_4 on trisilver phosphate.

In general, the properties of the higher acyl phosphates resemble those reported by Lipmann and Tuttle for acetyl phosphate but differ in degree. The higher acyl phosphates also possess striking colloidal properties not shared by the lower homologues. The enzymatic reactions of the higher fatty acid phosphates will be described in detail in a forthcoming report.

EXPERIMENTAL

Monopalmitylphosphoric Acid—In an ice-cold morter 7.52 gm. of ovendry Ag_3PO_4 were ground with 4.10 gm. (2.34 ml.) of 92 per cent H_3PO_4 (prepared by passing dry air through 85 per cent H_3PO_4 for 30 minutes at 150° or until a specific gravity of 1.77 was attained). The mixture was more finely dispersed after the addition of cold absolute ether and grinding to make a suspension (total volume of 40 ml.) of the gray or grayish yellow "monosilver phosphate." To it were added 11.1 gm. of palmityl chloride dissolved in 50 ml. of cold absolute ether. The solution was added dropwise with continual stirring and grinding with the pestle. After the addition was complete, the mixture was stirred an additional 5 to 10 minutes. The reaction mixture was centrifuged free of solid material and the supernatant (which contains the free monopalmitylphosphoric acid) was decanted into a 500 ml. suction flask. The residue was extracted with several portions of ether and the washings added to the original supernatant. The ether was removed *in vacuo*, leaving a white, sometimes crystalline solid. 300 to 400 ml. of hot benzene (50–60°) were added to the residue with swirling to yield a heavily turbid solution, which was then filtered, yielding a slightly turbid filtrate. This was placed in the cold room and allowed to cool to 6–8°. With slow cooling the monopalmitylphosphoric acid separated in crystalline form. The compound was collected on a sintered glass plate, washed with a little cold benzene, and dried *in vacuo*. The material

consists mainly of monopalmitylphosphoric acid with some free palmitic acid. Total phosphorus analysis of the dry material showed approximately 8.5 per cent P. To obtain the analytically pure compound it was recrystallized in such a manner as to free it from palmitic acid. This could be done by repeating the recrystallization from warm benzene several times. However, the differential solubility of the two compounds is much greater in ether-benzene mixtures, allowing recrystallization of the monopalmitylphosphoric acid to give analytically pure material in much less time, although at a considerable loss in yield. The crude material was dissolved in absolute ether (150 mg. per ml.). 5 volumes of benzene were added with mixing and the slightly turbid solution placed in an ice bath. The sides of the vessel were scratched with a glass rod to initiate crystallization. The monopalmitylphosphoric acid separated as glistening colorless platelets. It was filtered off on a sintered glass plate (sucking moist air through the substance should be avoided), washed with cold benzene, and dried *in vacuo* at room temperature. The yields were between 2 and 3.5 gm., or about 20 per cent of that calculated. The compound decomposed at 61–63°.

Analysis—$C_{16}H_{33}O_5P$. Calculated, P 9.22, C 57.13; found, P 9.21, C 57.20

The pure compound is unstable and absorbs atmospheric water, losing its crystalline structure with formation of palmitic acid and phosphoric acid. When kept in a vacuum desiccator over $CaCl_2$, it can be preserved for a week or so before decomposition is noticeable.

Monopalmitylphosphoric acid is soluble in water (see later), ether, ethanol, acetone, and ethyl acetate. It is only sparingly soluble in the less polar solvents (benzene, xylene, toluene, carbon tetrachloride).

If the compound was to be preserved for any length of time, it was usually converted to the disilver salt.

Disilver Palmityl Phosphate—2 gm. of recrystallized palmityl phosphate were dissolved in 50 ml. of 75 per cent ethanol and neutralized to pH 7 with NaOH. An excess of 25 per cent $AgNO_3$ solution was added, yielding a flocculent white precipitate. This was filtered off, washed with alcohol and ether, and dried *in vacuo* protected from light. A nearly quantitative yield of the greasy white disilver salt was obtained.

Analysis—$C_{16}H_{31}O_5PAg_2$. Calculated, Ag 39.22, P 5.64; found, Ag 39.31, P 5.77

Calcium Monopalmityl Phosphate—When treated with excess calcium acetate, an aqueous solution of disodium palmityl phosphate formed a flocculent precipitate of the calcium salt. The calcium and magnesium salts of palmityl phosphate are considerably more soluble than the corresponding salts of free palmitic acid.

Analysis—$C_{16}H_{31}O_5PCa$. Calculated, P 8.28; found, P 8.48

Barium Monopalmityl Phosphate—Barium ions form an exceedingly insoluble salt with aqueous solutions of the free acid.

Analysis—$C_{16}H_{31}O_4PBa$. Calculated, P 6.58; found, P 6.58

Distrychnine Palmityl Phosphate—When a dilute neutral solution of palmityl phosphate was treated with an equal volume of saturated strychnine sulfate solution and the mixture left at 0° overnight, an insoluble distrychnine salt was formed. This reagent may possibly be of analytical value, since it is capable of separating the acyl phosphate from inorganic phosphate. Other precipitants tested did not share this property.

Analysis—$C_{58}H_{75}O_9N_4P$. Calculated, P 3.09; found, P 3.33

Monooctanoyl Phosphate—A mixture of 7.52 gm. of Ag_3PO_4 and 4.10 gm. of 92 per cent H_3PO_4 was prepared as in the palmityl phosphate method. The mixture was suspended in 40 ml. of absolute ether. To the suspension were added 8.0 ml. of octanoyl chloride dissolved in 30 ml. of absolute ether. The solution was added dropwise, with constant stirring with the pestle. After addition of the octanoyl chloride was complete, the mixture was stirred for 5 more minutes and the AgCl removed by centrifugation. The precipitate was washed with a 50 ml. portion of ether and the supernatants then combined, filtered to remove any unsedimented AgCl, and evaporated to a thick creamy fluid under diminished pressure. This material contains free octanoylphosphoric acid, unchanged octanoyl chloride, free octanoic acid, and some inorganic phosphate, as well as silver (in the form of an ether-soluble silver salt). It was taken up in 100 ml. of ice-cold water, shaking being avoided, to yield a creamy, strongly acid solution of high foaming power. To this solution was added a great excess of 25 per cent $AgNO_3$ with thorough stirring, yielding a white precipitate containing the disilver salt of octanoyl phosphate, as well as some silver chloride and co-precipitated silver octanoate. It is free of inorganic phosphate (since even traces of Ag_3PO_4 will give a cream or yellow color). The precipitate was centrifuged off, the sediment packed to a small volume, and the supernatant drained off completely. The precipitate was then suspended in ice-cold water (100 ml.) and cold 1 N HNO_3 was added dropwise with shaking, dissolving most of the material and leaving a heavily turbid solution. This was immediately filtered by gravity through a previously wetted Whatman No. 40 paper, yielding a filtrate of only slight turbidity. The filtration removes AgCl and also much free octanoic acid, which is adsorbed on the paper. To the filtrate was then added 25 per cent $AgNO_3$ dropwise with *thorough stirring*, yielding a white, usually crystalline (platelets) precipitate. By precipitation in acid solution the octanoyl phosphate is separated from free octanoic acid and inorganic phosphate, which do not precipitate under

these conditions. After completeness of precipitation was assured, the silver salt was centrifuged off, washed with 1 volume of water, 95 per cent ethanol, and ether successively in the centrifuge, and then dried *in vacuo* over H_2SO_4, yielding a greasy white material of analytical purity. The yield was between 5 and 6 gm, or about 25 per cent over-all yield. *All operations involving the silver salt must be carried out in a darkened room to avoid the photochemical reduction of Ag, to which the salt is extremely susceptible.*

Analysis—$C_8H_{15}O_5PAg_2$. Calculated, Ag 49.27, P 7.08; found, Ag 49.40, P 6.91

Several methods for removal of the free octanoic acid, inorganic phosphate, etc., were examined critically but the method of precipitation by an excess of Ag in acid solution appeared simplest and most effective. For use in enzyme experiments the presence of these impurities in small amounts is immaterial, since the acyl phosphates are more or less quickly dephosphorylated by every mammalian tissue preparation studied.

The compound is perfectly stable if kept in a vacuum desiccator shielded from light. It can be converted into the sodium salt by shaking with a 10 per cent excess of the calculated amount of NaCl. The AgCl is removed by high speed centrifugation to give a water-clear neutral solution. The concentration can readily be established by means of an inorganic phosphate determination or the colorimetric acyl phosphate determination of Lipmann and Tuttle (8).

Barium, calcium, and strychnine salts were also prepared. The analytical data are given in Table I.

The phosphates of several other higher fatty acids were prepared by the procedures outlined. Crystallization of the free acids could be effected from ether-benzene mixtures if the acid contained 10 or more carbons. The losses were great with the 10- and 12-carbon acids but became progressively less with an increase in chain length.

Solubility and Colloidal Behavior—The 8- and 16-carbon compounds dissolve in water to give acid solutions of high foaming power. This is in direct contrast to the insolubility in water of free octanoic and palmitic acids, especially the latter. The solutions are strong emulsifying agents. The change from the insolubility of free palmitic acid to the pronounced hydrophilic properties of palmityl phosphate was also demonstrated by the colloidal properties of the latter. When crystals of palmityl phosphate were moistened with water and triturated, the substance swelled and assumed a gel-like form. On dilution with water, a solution having a strong Tyndall effect was obtained. Palmityl phosphate could be salted-out by 0.5 saturation with $(NH_4)_2SO_4$.

Crude measurements made with a stalagmometer and the du Noüy tensiometer on solutions of the acyl phosphates and corresponding fatty

acids at the same concentration and pH showed that although the acyl phosphates lowered surface tension, as was expected, they were not as active in this respect as were the fatty acids.

The introduction of the highly polar phosphate group into the fatty acid molecule evidently conferred striking hydrophilic properties on these compounds. The change in properties of the fatty acid on phosphorylation brings to mind similar properties exhibited by lecithin, for example, in contrast to those of a triglyceride.

Apparent Dissociation Constants—Although colloidal solutions of the sort described above obviously deviate widely from the ideal, the apparent dissociation constants at 25° were calculated from titration curves constructed from pH data obtained potentiometrically with the glass electrode and Cameron pH meter. Solutions of octanoyl and palmityl phosphate in concentrations from 0.001 to 0.01 M were either made up by weight or obtained by metathesis in CO_2-free water. · Concentrations were checked by

TABLE I

Analytical Data on Salts of Octanoyl Phosphate

Salt	Formula	P calculated	P found
		per cent	*per cent*
Ca	$C_8H_{15}O_5PCa$	11.83	11.98
Ba	$C_8H_{15}O_5PBa$	8.63	8.72
Strychnine	$C_{50}H_{59}O_9N_4P$	3.48	3.80

suitable analytical methods. Titrations from both the acid and basic side were performed. The dissociation constants given were calculated by methods outlined by Van Slyke (9) or obtained graphically. They represent average figures. The titration curves showed two points of inflection corresponding to primary and secondary stages of dissociation. pK_1 and pK_2 values for the two compounds are shown in Table II, together with values for orthophosphoric acid, phosphocreatine, and glucose-1-phosphate for comparison.

It is well known that esterification of phosphoric acid with certain organic compounds causes a strengthening of its acidic properties (10, 11). In the case of the higher fatty acid phosphates here studied, this did not appear to be true of the first stage of dissociation but it did hold true for the physiologically important second stage. Some reservations must be made on the accuracy of the measurements because of the difficulty of reproducing the colloidal conditions (at least in the case of the palmityl phosphate) and the slight spontaneous hydrolysis of the acyl phosphates during the titrations.

Stability of Aqueous Solutions—Lipmann and Tuttle have studied the

stability of acetyl phosphate in aqueous solution as a function of pH (6). The stability of octanoyl and palmityl phosphate followed the same pattern with regard to pH but was somewhat greater than that of acetyl phosphate. The course of the hydrolytic reaction was followed manometrically by measuring the displacement of CO_2 from a bicarbonate buffer at neutral reactions. This was made possible because the hydrolysis of the acyl phosphates liberates a new acid group according to the equation

(2) $RCOOPO_3H_2 + H_2O \rightarrow RCOOH + H_3PO_4$

At pH 7.4 and 37° both octanoyl phosphate and palmityl phosphate possessed a half life of some 12 hours (acetyl phosphate, 3 hours) (see Table III). Calculations employing the pK_2 values determined in the previous section and the pK_2 values of orthophosphoric acid in the Henderson-Hasselbalch equation showed that in the case of octanoyl phosphate com-

TABLE II

Apparent Dissociation Constants

Compound	pK_1	pK_2
Monooctanoylphosphoric acid.....................	3.00	5.4
Monopalmitylphosphoric "	3.37	6.2
Glucose-1-phosphoric acid*......................	1.10	6.13
Phosphocreatine†................................		4.58
Orthophosphoric acid‡...........................	1.95	6.83

   * Taken from Cori, Colowick, and Cori (10).
   † Taken from Fiske and Subbarow (11).
   ‡ Taken from Van Slyke (9).

plete hydrolysis at pH 7.4 should liberate approximately 0.76 equivalent of CO_2, palmityl phosphate about 0.82 equivalent. The yields of CO_2 obtained were 0.755 and 0.78 equivalent respectively, which are in fair agreement considering the inaccuracies involved and the lower temperature in the determination of the dissociation constants. The hydrolytic reaction followed the first order law in both cases. The deviations in the case of palmityl phosphate were probably due to the precipitation of free palmitic acid which occurred, leading to low CO_2 values.

Behavior toward Acid Molybdate Solutions—Lipmann and Tuttle (6) and Fiske and Subbarow (11) have found that the phosphorus of acetyl phosphate and phosphocreatine respectively appears as inorganic P when analyzed by the Fiske and Subbarow molybdate colorimetric method (12). This hydrolysis occurs within the usual color development period. This property is also shared by the acyl phosphates here studied. The rate of color development (aminonaphtholsulfonic acid being used as the reducing

agent) in the presence of these compounds compared to the rate in the presence of orthophosphate was followed in a Coleman spectrophotometer. The rate of development of color from the acyl phosphates lagged behind that of inorganic P by only a very small interval. This was difficult to determine accurately in the case of palmityl phosphate, because the liberation of free palmitic acid produced a slight turbidity in the solutions. Maximum absorption values for the acyl phosphates were obtained well within the usual color development time (10 to 15 minutes). The reaction

TABLE III

Rate of Spontaneous Hydrolysis of Octanoyl and Palmityl Phosphate at pH 7.4 and 37°

The Warburg vessels contained 1.00 ml. of the acyl phosphate (or H_2O blank) and 2.00 ml. of 0.03 M $NaHCO_3$ in the main compartment. The side arm contained the residue of 0.40 ml. of 0.0125 N tartaric acid dried *in situ* at 75°. The gas phase was 5 per cent CO_2 in nitrogen. The retention of CO_2 by foreign buffer was found by allowing the tartaric acid to dissolve in reaction media at the completion of the hydrolysis and comparing with a blank determination without acyl phosphate. Octanoyl phosphate added, 3.25×10^{-6} mole; palmityl phosphate, 2.23×10^{-6}.

Time	Octanoyl phosphate			Palmityl phosphate		
	CO_2 displaced	Hydrolysis	$K \times 10^{-4}$	CO_2 displaced	Hydrolysis	$K \times 10^{-4}$
min.	*c.mm.*	*per cent*	*min.*$^{-1}$	*c.mm.*	*per cent*	*min.*$^{-1}$
0	0	0		0	0	
96	4.8	8.7	9.46			
210	9.8	17.8	9.36	7.0	17.9	9.42
390	17.0	30.9	9.52	13.0	33.3	10.4
720	27.0	49.1	9.38	19.7	50.2	9.76
1320	39.0	70.9	9.35	25.5	65.4	8.20
3900	55.0	100		39.0	100	
Average			9.41			9.45

mixture contained 0.4 ml. of 10 N H_2SO_4, 0.8 ml. of 2.5 per cent ammonium molybdate, and 0.4 ml. of reducing agent in a 10.0 ml. total volume.

Enzymatic Hydrolysis of Acyl Phosphates—Both the acyl phosphates described here, as well as acetyl phosphate, were quickly dephosphorylated by homogenates or extracts of different rat tissues. The reaction was followed manometrically by measuring the displacement of CO_2 from a bicarbonate buffer.

Since adenine nucleotides are required in the oxidation of pyruvic acid in both bacterial extracts (13) and in brain dispersions (14), in the oxidation of α-ketoglutarate in heart muscle preparations (15), in the oxidation of glyceraldehyde phosphate (16), and also in fatty acid oxidation (1), it would appear that the nucleotides may be required as obligatory phosphate ac-

ceptors for some intermediate compound formed in oxidation. At least this has been demonstrated in the case of a bacterial oxidation of pyruvate (13) and in glyceraldehyde phosphate oxidation (16). However, Ochoa has noted (15) that succinyl phosphate was rapidly dephosphorylated by his α-ketoglutaric dehydrogenase preparation in the absence of adenine nucleotide, making it seem unlikely that an obligatory transphosphorylation was involved in the need for adenine nucleotide in α-ketoglutarate oxidation, if succinyl phosphate is the actual intermediate in this oxidation.

To test the assumption that a transphosphorylation to adenine nucleotide is required in the dephosphorylation of fatty acid phosphates, a saline extract of rat liver was very thoroughly dialyzed (inside and outside stirring) against several changes of saline at 4° for 4 days. The hydrolytic activity dropped about 45 per cent in this period, compared to an undialyzed sample stored for the same length of time. The activity was almost completely restored by the addition of Mg^{++}. Adenylic acid or adenosine diphosphate did not stimulate the hydrolysis. Fluoride inhibited the hydrolysis, as was expected. This extract, as well as most tissue preparations, dephosphorylated the acyl phosphates faster than adenosine triphosphate. Extracts of acetone powders of rat liver contained a potent acylphosphatase but no adenosinetriphosphatase. The acylphosphatase activity was found to be quite resistant to acid, a treatment which inactivated completely phosphatase activity toward several other substrates. It would appear that a specific phosphatase for the acyl phosphate linkage is present in extracts of rat liver, and that the adenylic acid system is not involved in its action.

SUMMARY

1. Monopalmitylphosphoric acid and monooctanoylphosphoric acid (mixed anhydrides of phosphoric acid and the fatty acid) have been synthesized by the reaction of the proper acyl chloride with monosilver phosphate. The methods developed may be used in the synthesis of other higher fatty acid phosphates.

2. Silver, calcium, barium, and strychnine salts of these compounds were obtained.

3. The introduction of the phosphate group into the fatty acid molecule causes it to assume the properties of a much more soluble, more hydrophilic substance.

4. The compounds are relatively unstable in aqueous solution, but are more stable than either acetyl phosphate or 1,3-diphosphoglyceric acid.

5. Phosphatases present in mammalian tissues quickly hydrolyze the fatty acid phosphates. The adenylic acid system is not an obligatory phosphate acceptor in the dephosphorylation of the fatty acid phosphates.

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THE EFFECT OF THE DIETARY LEVEL OF METHIONINE ON THE RATE OF TRANSMETHYLATION REACTIONS IN VIVO*

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During a study of the rôle of methionine in transmethylation, we became interested in the influence of the dietary level of deuteriomethionine on the rate at which deuteriomethyl groups appear in the tissue choline and creatine. To investigate this point, a paired feeding experiment was set up in which diets free of choline and creatine and containing 0.7 and 1.4 per cent deuteriomethionine ($CD_3SCH_2CH_2CHNH_2COOH$) respectively were used. Two pairs of young rats, each rat weighing about 65 gm., were placed on each of these diets for 7 days. At the end of this period, the animals were sacrificed and the choline and creatine of their tissues were isolated. Deuterium analyses indicated that the amount of isotope in the isolated compounds was dependent upon the amount of deuteriomethionine in the diet. The choline and creatine isolated from the tissues of the two rats fed the diet containing 1.4 per cent deuteriomethionine contained almost twice as much deuterium as the choline and creatine isolated from the tissues of their litter mates fed the 0.7 per cent deuteriomethionine diet.

If the dietary level of deuteriomethionine had been without effect on the amount of deuterium found in the isolated choline and creatine, one could, of course, have concluded that the dietary level does not affect the rate of transmethylation. Unfortunately, the converse does not necessarily follow. The observed difference in the amount of deuterium which appeared in the choline and creatine at the two different dietary levels may be due to different isotopic concentrations of the methionine which participates in the transmethylation reaction rather than to different rates of transmethylation or even to a combination of both these effects. Since the procedures which have been worked out (1) for the determination of the deuterium content of tissue methionine are not quantitative, it was not possible to determine the actual deuteriomethyl content of the methionine in the tissues which would reflect to some extent the deuteriomethyl content of the methionine involved in the transmethylation reaction.

To distinguish between the possible alternatives necessitated another type of experimental approach. The following experiment was set up to eliminate the isotopic concentration of the methionine as a factor.

Five adult male rats were fed deuteriocreatine for a 3 week period in addition to their normal casein diet. The feeding of deuteriocreatine was then discontinued. Two of the rats were fed a diet which contained about 0.7 per cent methionine in the form of casein, while the other three rats were fed the same amount of casein supplemented with sufficient *dl*-methionine to bring the total methionine level to about 1.4 per cent. The rates of disappearance of the deuteriocreatine at the two dietary levels of ordinary methionine were followed for 40 days by isolating samples of creatinine from the urine collected from each group at intervals. Since deuteriocreatinine excreted on a creatine-creatinine-free diet reflects directly the isotope content of tissue creatine (2), the changes in isotope concentration in the urinary creatinine were used here to follow the changes in tissue creatine throughout the experimental period. Finally the rats were sacrificed and creatine was isolated from the tissues of each animal.

EXPERIMENTAL

$$
\begin{array}{c}
NH_2 \\
| \\
C{=}NH \\
|
\end{array}
$$

Synthesis of Deuteriocreatine (CD_3NCH_2COOH)—Deuteriosarcosine hydrochloride $(CD_3NHCH_2COOH \cdot HCl)$ was prepared from deuteriomethyl iodide (2) and *p*-toluenesulfonylglycine by the method of Fischer and Bergmann (3). A solution of 4.4 gm. of this sarcosine hydrochloride in 14 cc. of water was treated with 4 cc. of concentrated ammonium hydroxide and 2.8 gm. of cyanamide dissolved in 10 cc. of water. The reaction mixture was allowed to stand at room temperature for 3 days, and the deuteriocreatine hydrate which crystallized was then filtered off. The creatine hydrate was recrystallized from water and dried to constant weight in an oven at 100° to convert it to deuteriocreatine. 2.84 gm. of the product were obtained.

Analysis—Calculated,[1] N 31.60; found, N 31.73

Deuterium analysis showed 62.7 atom per cent deuterium in the methyl group.

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[1] Calculated values based on increased molecular weight due to deuterium in the molecule.

Feeding of Deuteriomethionine at Two Different Levels—Four young female rats were placed on synthetic diets containing deuteriomethionine (2); one pair of rats received a diet containing 0.7 per cent deuteriomethionine and the other pair received a diet containing 1.4 per cent deuteriomethionine. The deuteriomethionine contained 16.7 atom per cent deuterium in the methyl group. The diets were otherwise identical and had the following percentage composition: amino acid mixture (4) 21.3, dextrin 25.7, sucrose 15.0, salt mixture (5) 4.0, agar 2.0, corn oil (Mazola) containing vitamins A, D, E, and K (4) 30.0, and deuteriomethionine plus dextrin 2.0. In addition, each rat was given daily two 0.5 cc. portions of an aqueous solution of B vitamins having the following composition (in

TABLE I

Paired Feeding Experiment with Deuteriomethionine

Rats fed deuteriomethionine (16.7 atom per cent deuterium in the methyl group) for 7 days.

Rat No.	Change in body weight	Level of deuteriomethionine in diet	Total deuteriomethionine ingested	Tissue choline Deuterium in isolated choline chloroplatinate	Tissue creatine Deuterium in isolated creatinine K picrate
	gm.	*per cent*	*gm.*	*atom per cent*	*atom per cent*
1748	68–78	0.7*	0.312	1.64 ± 0.11	0.52 ± 0.08
1749	63–73	0.7	0.319	1.75 ± 0.17	0.60 ± 0.09
1750	67–69	1.4	0.546*	2.49 ± 0.10	0.76 ± 0.09
1751	65–75	1.4	0.623	3.03 ± 0.09	0.89 ± 0.08

* For the first 6 days Rat 1750 ingested approximately the same amount of deuteriomethionine as Rat 1751, but, on the 7th day, the total food intake of Rat 1750 was considerably below that of Rat 1751.

mg. per 1 cc.): 0.02 each of thiamine chloride, nicotinic acid, pyridoxine hydrochloride, and riboflavin, 0.2 of *dl*-calcium pantothenate, and 25 of choline-free ryzamin-B (6). During the 7 day experimental period, the food intake was controlled. The amount of deuteriomethionine ingested by each rat is given in Table I. It should be noted that the amount of deuteriomethionine ingested by Rat 1750 was somewhat less than that ingested by Rat 1751, the other member of the pair. This was due to the fact that Rat 1750 ate considerably less than Rat 1751 on the final day of the experiment.

After 7 days the animals were sacrificed and choline and creatine were isolated (2) from the tissues of each animal as choline chloroplatinate and creatinine potassium picrate respectively. The purity of the picrates was determined colorimetrically by the Jaffe reaction.

Analysis—Choline chloroplatinate

$$\begin{array}{llll}
\text{Rat 1748.} & \text{Calculated,[1]} & \text{Pt 31.6; found,} & \text{Pt 31.6} \\
\text{`` 1749} & \text{``} & \text{`` 31.6;} & \text{`` `` 31.6} \\
\text{`` 1750} & \text{``} & \text{`` 31.6;} & \text{`` `` 31.5} \\
\text{`` 1751} & \text{``} & \text{`` 31.6;} & \text{`` `` 31.6}
\end{array}$$

All the isolated compounds were analyzed for deuterium and the results of these analyses are given in Table I.

Feeding of Ordinary Methionine at Two Different Levels—Five male rats, weighing from 290 to 390 gm., were placed in individual cages arranged for urine collection and fed a casein diet having the following percentage composition: vitamin-free casein (Smaco) 20, salt mixture (5) 4, Crisco 19,

TABLE II

Deuteriocreatine Experiment

Rats fed 5 mg. per 100 gm. of body weight per day of deuteriocreatine (62.7 atom per cent deuterium in the methyl group) for 21 days prior to initial urine collection. Group A, two rats; Group B, three rats.

	Days after last deuteriocreatine feeding; period of urine collection	Deuterium in material isolated	
		Group A (0.7 per cent methionine diet)	Group B (1.4 per cent methionine diet)
		atom per cent	*atom per cent*
Urinary creatinine	0–3	4.15 ± 0.12	3.90 ± 0.10
	4–6	4.00 ± 0.12	3.94 ± 0.14
	11–13	3.61 ± 0.16	3.55 ± 0.16
	18–20	3.14 ± 0.13	3.08 ± 0.12
	25–27	2.47 ± 0.13	2.38 ± 0.11
	32–34	2.14 ± 0.18	2.10 ± 0.10
Tissue creatine	40	2.06 ± 0.07 (Rat 1)	2.01 ± 0.10 (Rat 3)
		1.99 ± 0.07 (`` 2)	2.23 ± 0.10 (`` 4)
			2.07 ± 0.08 (`` 5)

corn oil (Mazola) 1, and sucrose 56. The fat-soluble vitamins were mixed in the diet and the water-soluble vitamins of the B complex were fed in aqueous solution, as described above. To the aqueous vitamin solution was added sufficient deuteriocreatine (62.7 atom per cent deuterium in the methyl group) to give each rat 5 mg. per 100 gm. of body weight per day. This diet and vitamin supplement were fed for 3 weeks. The deuteriocreatine feeding then was discontinued and the rats were divided into two groups: Group A, with two rats, and Group B with three rats. Group A was continued on the 20 per cent casein diet, which contained approximately 0.7 per cent methionine. Group B was given the 20 per cent casein diet to which was added 0.7 gm. of methionine per 100 gm. of diet. Thus, Group B received a diet containing approximately 1.4 per

cent methionine. The rats were continued on these creatine-free diets for 40 days, and the daily food intake was controlled in order that the rats in Group B should ingest at least twice as much methionine as did the rats in Group A.

Urine was collected over the first 72 hours after the feeding of deuterio-creatine had been discontinued and then was collected over 48 hour periods at weekly intervals, as indicated in Table II. The urine collected from the rats in each group was pooled and the total urinary creatinine was determined colorimetrically by the Jaffe reaction. There was no significant difference between the amounts of creatinine excreted per 100 gm. of body weight by the rats in the two groups. Creatinine was isolated from the pooled urine as creatinine potassium picrate (7).

On the 40th day after deuteriocreatine feeding had been discontinued, the animals were sacrificed, and the creatine was isolated from the tissues of each rat (2). The deuterium contents of all the isolated compounds are given in Table II.

DISCUSSION

The use of data concerning the rate of appearance of one isotopic compound after the feeding of another isotopic compound for conclusions regarding the rate of synthesis of the former is fraught with many difficulties. At best, one is measuring the difference between an over-all rate of formation and the rate of disappearance of the compound. Nevertheless, when the rate of appearance of deuterium in choline and creatine varies, with a variation in the dietary level of deuteriomethionine, as shown in Table I, at least one of the rate steps involved must depend upon the dietary level of methionine.

As already pointed out in the introduction, the results of the paired feeding experiments with 0.7 per cent and 1.4 per cent deuteriomethionine diets respectively could not indicate which step or steps in the over-all series of reactions were dependent on the dietary level of methionine. Should it be the rate of transmethylation from the methionine of the body to choline and creatine which increases as the level of dietary methionine increases, it would follow that the amount of choline and creatine synthesized in a given period of time can be altered merely by changing the amount of methyl donor in the diet. Such a variation in rate of synthesis is particularly improbable for creatine because of the constancy of the total amount of creatine in the body and the concomitant constancy of creatinine excretion. The mere possibility of such an interpretation was, however, sufficiently startling in view of current theories to warrant the further investigation involved in the second series of experiments.

Of course the other explanation, namely that the difference in the

amount of deuterium which appears in the choline and creatine at the two dietary levels of methionine is due only to the different rates of appearance of deuteriomethionine in the methionine which participates in the transmethylation reactions, leads to a more orthodox interpretation. If this explanation is correct, then the deuterium content of the choline and creatine from the pair of rats fed the 1.4 per cent deuteriomethionine diet will, of necessity, be greater than that of the pair fed the 0.7 per cent diet, even though the rates of transmethylation remain the same.

It is sometimes possible to choose experimental conditions such that the interpretation of data concerning rates is simplified and the necessity of introducing many assumptions is obviated. Such conditions are illustrated in the experiment of Bloch, Schoenheimer, and Rittenberg (8) in their study of the rate of creatine synthesis. These authors followed the rate of disappearance of N^{15} creatinine from the urine of rats after the feeding of N^{15} creatine had been discontinued. Since it had been shown that (a) the total amount of creatine in the animal body remains constant, (b) the nitrogen of creatine does not participate in any further anabolic reactions, and (c) the rate of excretion of creatinine remains constant, then the rate of disappearance of isotopic creatine is equivalent to the rate of synthesis of creatine.

Unfortunately, in the case of choline, it is not possible to study the rate of synthesis, i.e. the rate of methylation of ethanolamine, directly as in the case of creatine. To follow the rate of synthesis by labeling the methyl group is impossible since transmethylation from methionine to choline is reversible. Nor is it possible to follow the rate of synthesis with N^{15}, for, although the total amount of choline apparently remains constant even in choline deficiency (9, 10), the rate of choline degradation is not known to remain constant.

Since the methyl group of creatine, unlike that of choline, does not enter any further transmethylation reactions (11), it is possible to study the rate of transmethylation to creatine in a manner analogous to the N^{15} study with creatine (8), simply by following the rate of disappearance of deuterium from tissue creatine which has been labeled by the feeding of deuteriocreatine. By the use of this technique, the effect of various factors on the rate of transmethylation to creatine may be investigated. In particular, the effect of different dietary levels of methionine on the rate of creatine synthesis could be followed.

If the rate of creatine formation does increase with the increase in the dietary level of the precursor of the methyl group, deuteriocreatine should disappear more rapidly from the tissue creatine of the three rats fed the larger amount of methionine. This was not the case. As shown in Table

II, there was no difference between the two groups with respect to the deuterium content of the urinary creatinine samples isolated at any given time during the 40 day experimental period. Nor was there any difference between the two groups in the deuterium content of the tissue creatine samples isolated at the end of the experimental period. Furthermore, quantitative creatinine determinations on the urines from the two groups showed that the same amount of creatinine was being excreted per 100 gm. of body weight by both groups. It may be concluded, therefore, that the
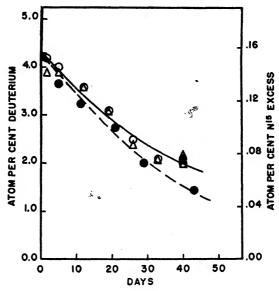
Fig. 1. A comparison of the rates of disappearance of the isotope in urinary creatinine obtained after feeding deuteriocreatine and N^{15} creatine respectively. $\bigcirc$, atom per cent deuterium in urinary creatinine, Group A; $\triangle$, atom per cent deuterium in urinary creatinine, Group B; $\bullet$, atom per cent N^{15} excess in urinary creatinine (8).

rate of transmethylation to creatine, at least in the adult rat, is independent of the dietary level of methionine.

As stated above, our experiment with deuteriocreatine was similar to that of Bloch and coworkers (8) in that the tissue creatine was labeled by the feeding of isotopic creatine and the disappearance of the isotope from the urinary creatinine was followed. Whereas deuteriocreatine was used in our laboratory, Bloch, Schoenheimer, and Rittenberg used creatine containing N^{15}. It is now possible to compare these two independent sets of data dealing with the rate of disappearance and simultaneous synthesis

of tissue creatine. In Fig. 1, the N^{15} concentrations of creatinine samples, obtained after the feeding of isotopic creatine had been discontinued (8), have been plotted for comparison with the deuterium concentrations of the creatinine samples obtained in our experiments summarized in Table II. The half time arrived at from the N^{15} data was 29 days, while that from the present data is approximately 36 days. Although these are not in complete agreement, they are of the same order of magnitude and probably indicate an experimental variation rather than a different metabolic fate of the methyl and guanidoacetic acid portions of the creatine molecule.

SUMMARY

A paired feeding experiment was carried out with immature rats on diets containing 0.7 or 1.4 per cent deuteriomethionine ($CD_3SCH_2CH_2CHNH_2$-$COOH$). Analysis of the tissue choline and creatine indicated that the deuterium content of these tissue constituents is dependent upon the amount of deuteriomethionine ingested.

After tissue creatine had been labeled with deuterium by the feeding of

$$NH_2$$
$$|$$
$$C=NH$$
$$|$$

deuteriocreatine (CD_3NCH_2COOH), creatine-free diets containing 0.7 or 1.4 per cent ordinary methionine were fed to two groups of adult rats. The deuterium content of the creatinine isolated at intervals from the urine of each group was determined. The level of methionine in the diet was found to have no effect on the rate at which deuterium disappeared from the urinary creatinine.

It was concluded that the rate of methyl transfer from methionine is not proportional to the level of methionine in the diet.

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THE ULTRAVIOLET IRRADIATION OF QUININE

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Although various investigators have recognized that the exposure of dilute solutions of cinchona alkaloids to light of short wave-lengths destroys the alkaloidal structure, neither the nature of this photochemical reaction nor its products have been established. The conclusions have been controversial in many respects. In one case (16) it was stated that the rate of reaction is reciprocally related to the concentration of oxygen in the solution, whereas in another (4) the presence or absence of oxygen was said to have no effect. Contradictory claims regarding the change of the pH of the solution during irradiation have appeared (4, 9). Others (6, 8, 14, 15) have suggested that an isomerization of quinine to quinotoxine is the result of the irradiation of this cinchona alkaloid. It has been established that this isomerization does occur to some extent when the alkaloid is dissolved in one of a number of organic acids and irradiated (2). Still another interpretation is that a dimerization takes place through the vinyl group on each of 2 molecules (4).

The objectives of this investigation have been the definition of the photodecomposition of quinine, under specified conditions of irradiation, by analytical methods which have not been applied heretofore and the biological evaluation of the irradiation products by a determination of their antimalarial activity. The results of the latter constitute a separate report.[1] Certain conclusions have evolved which are incidental to the primary objectives and which bear on some of the controversial claims that have been previously cited.

EXPERIMENTAL

Irradiation with Direct Sunlight

Solutions—Quinine dihydrochloride, which had been dried to constant weight *in vacuo* over phosphoric anhydride and shown to be pure by a determination of its specific rotation (1), was used in the preparation of a stock solution which contained 25 gm. of quinine as the free base per liter of the aqueous salt solution. Standard solutions, each of which contained

5 mg. of quinine per ml. in 0.0005 N NaOH, water, and 0.030, 0.060, 0.125, 0.250, 0.500, 1.00, and 2.00 N HCl, were prepared by dilution of 100 ml. of the stock to 500 ml. with the calculated volume of 0.1 N NaOH and alcohol, water, or HCl of convenient normality and water. The standard with an alkaline reaction was prepared in 50 per cent alcohol in order to maintain complete solution of the free base. From each standard, ten serially numbered containers were filled and tightly stoppered. The containers consisted of 50 ml. Pyrex flasks with rubber stoppers and clear soft glass vials with paraffined cork stoppers. Each of the two kinds of containers was arranged alternately throughout the series.

Irradiation—With the exception of one from each series which was stored in a dark cabinet to serve as a control, all flasks and vials were placed outside on the sills of south windows. The location provided direct sunlight at practically all hours of the day. Daily irradiation was continuous for each until the time of its removal from sunlight for analysis. An hourly record of bright sun was kept for exposures of 10 days or less and cloudy intervals were disregarded. The accumulation of 10 hours of bright sun was recorded as 1 day. Exposures in excess of 10 days were recorded according to the calendar.

Observations—A member from each series was removed simultaneously at consecutively greater intervals of exposure as follows: 0.5, 1.0, 3.0, 10, 35, 105, and 275 days. Each solution was analyzed routinely by three procedures and the result for each procedure was expressed in percentage of the original concentration of quinine which the solution contained before irradiation.

A 1 ml. sample of the irradiated solution was placed by pipette in a 250 ml. volumetric flask and diluted to the mark with the calculated amount of dilute standard HCl and water so that the resulting solution contained 0.030 N HCl. An Evelyn photoelectric colorimeter was used to analyze this diluted solution of the irradiated quinine according to the procedure by Kyker, Webb, and Andrews (11) for the estimation of quinine. Determinations were made both before and after samples were subjected to continuous ether extraction. Triplicate samples were determined in every case. The direct estimation of quinine was carried out by pipetting 2 ml. samples of the solution into each of three colorimeter tubes, adding 8 ml. of 0.030 N HCl, mixing, preparing a silicotungstate turbidity, and measuring the density of the turbidity in the manner which is described under *macroprocedure* in the above method. In the event that no chemical changes resulted from the irradiation, the density of the turbidity should correspond to a concentration of 4 mg. of quinine per liter. The alternate procedure for the analysis of this diluted solution consisted of placing a 7 ml. sample in a continuous extractor, rendering it alkaline with 3 ml. of 0.1 N NaOH,

and dissolving the ether-extractable material in 35 ml. of 0.030 N HCl from which solution three 10 ml. samples were taken for the final estimation. The dilution factor is of the same order as that which is prescribed in the first procedure and a concentration of 4 mg. of quinine per liter would be indicated for samples containing unchanged quinine.

The specific optical rotation was determined for each of the irradiated solutions. All determinations were made with a Schmidt and Haensch half shadow polarimeter which reads to ±0.01°. Monochromatic D light from an electric sodium vapor lamp was employed and all readings were made at 28° ± 1°.

Irradiation with Ultraviolet Lamp

Solutions—The following solutions were prepared and irradiated: quinine dihydrochloride in 2.00 N HCl, 2 and 6 gm. per liter; quinine sulfate dihydrate in 0.100 N H_2SO_4, 6 gm. per liter; and quinidine sulfate in 0.100 N H_2SO_4, 0.02 gm. per liter.

Irradiation—A 125 watt mercury arc lamp[2] with a $1\frac{1}{2}$ inch quartz filament was used in all cases. The solutions were irradiated in a disk-shaped quartz cell (dimensions, 9 mm. thickness, 72 mm. diameter), equipped with a quartz stopper, which was placed across the path of radiation at a horizontal distance of 12 inches from the filament. The heat from the lamp kept the cell and contents at 30–32° during operation. No forced ventilation was provided. In each experiment, the cell was filled to its capacity, placed in the path of radiation, and at specified intervals 1 ml. samples were removed for analysis.

Observations—The samples were diluted to an appropriate concentration of the alkaloid in 0.030 N HCl or 0.100 N H_2SO_4, from which aliquots were analyzed by one or more of four procedures. Two of these procedures were similar to the silicotungstate turbidimetry which is described above for the products of sunlight irradiation. The other two employed the fluorescence of the sulfate of the alkaloid, which was measured on samples before and after extraction. The instrumentation consisted of a Coleman electronic photofluorometer. The extraction which was applied before either the turbidimetric or fluorometric determination was a continuous process with chloroform (12) rather than the previously used continuous ether extraction.

DISCUSSION

Observations on the solutions which were irradiated by sunlight are presented in Fig. 1. An evaluation of the stability of these solutions depends

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[2] The lamp which was used is an Alpine Sun Burner type, a standard model, constructed by the Hanovia Chemical and Manufacturing Company, Newark, New Jersey.
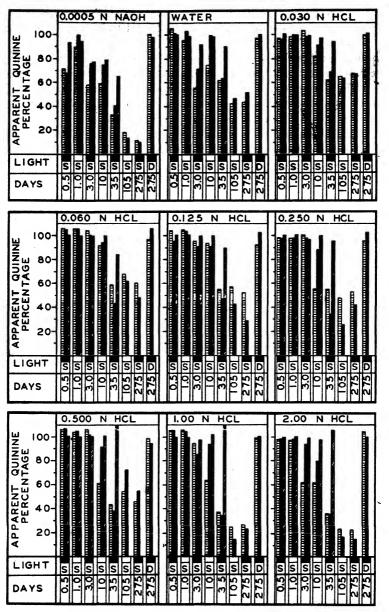
Fig. 1. The irradiation of quinine by sunlight. Within each column the horizontally hatched, the solid, and the 60° hatched posts represent respectively the data from determinations by direct silicotungstate turbidimetry, by ether extraction and silicotungstate turbidimetry, and by polariscopic measurements. The columns resting on solid pillars pertain to solutions which were irradiated in Pyrex containers, and those resting on open pillars pertain to soft glass containers. S signifies continuous exposure to sunlight and D signifies continuous storage in a dark cabinet.

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considerably on the method of analysis which is chosen to evaluate the concentration of the irradiated solution in terms of the original concentration of quinine. Polariscopic measurements reflect the least change of all the methods which were used. These measurements could not be made on any of the solutions which were irradiated longer than 35 days, because the discoloration which accompanied photodecomposition became so intense that reliable readings were impossible. Two of the solutions (0.500 N
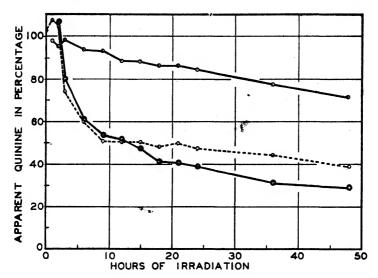
FIG. 2. Ultraviolet irradiation of quinine in 2 N HCl by the mercury arc lamp. The curves represent the same irradiation product and correspond respectively to the following analytical methods and treatments which were employed: broken line and single circles, direct turbidimetry; solid line and double circles, turbidimetry of material extractable with chloroform; solid line and single circles, fluorometry of material extractable with chloroform.

and 1.00 N hydrochloric acid) showed a large increase in the apparent quinine content after 35 days exposure. These are represented by broken posts in Fig. 1; the observed rotation was equivalent in each case to 133 per cent of the unexposed quinine. Considerable destruction of quinine was shown in all of the solutions by silicotungstate turbidimetry both when this method was applied directly to the irradiated solutions and when the alkaloidal components were separated previously by extraction.

Each of the analytical procedures indicated more decomposition in the dilute sodium hydroxide solution than in any of the other solutions. The increase in the stability of the alkaloid in 0.030 N acid over that in 0.0005 N alkali was pronounced. The stability continued to increase with the con-

centration of the acid to 0.060 N, declined slowly to 0.250 N acid, and rapidly to 2.00 N hydrochloric acid.

In the higher concentration of acid in which the decomposition is more pronounced, the quantitative progression of the reaction is smoother when those samples which were in Pyrex containers are considered as a series and compared with the series of samples in the soft glass containers. The soft glass showed more opacity to the effective wave-lengths of light from the sun.

The samples in the various acid solutions which showed no significant change in quinine content by any of the methods of analysis did undergo some chemical reaction. The basis for this claim is the discoloration which appeared very early during the irradiation and gradually became quite intense before any significant change could be confirmed by analysis. A yellow or amber color appeared first in the solutions of both high and low acid concentrations. The intermediate solutions first showed a pink color. The former progressed to a brownish turbid state and the latter to a deep wine-red color. The control samples which were stored in the dark retained both their original appearance and their original content of quinine, as measured by each of the analytical methods.

The use of an artificial source of irradiation provided a more quantitative means of treatment, a more rapid rate of decomposition, and results which are qualitatively similar to those from irradiation by sunlight. These results which are presented in Fig. 2 were derived from a study of solutions which contained 2 gm. of quinine dihydrochloride, as the free base, in 2.00 N hydrochloric acid. These solutions contained the same amount of acid and 40 per cent as much quinine as the solution which underwent the greatest change by exposure to sunlight. Any differences in the observations of the artificially irradiated solutions which depend on the analytical procedures are small when turbidimetric analyses, before and after extraction of the alkaloidal components with chloroform, are compared. The divergence of the two curves in Fig. 2 which provide this comparison is greater beyond 20 hours irradiation, however, than the combined error of the two methods. By fluorometric analysis the concentration of quinine decreases almost linearly and more slowly than by turbidimetry. Polariscopic measurements were made but are not included in Fig. 2 because they indicated only slight changes in the composition of the solutions. After as much as 50 per cent of the quinine was shown to be decomposed by turbidimetry, there remained from 96 to 100 per cent of the original quinine according to polariscopic determinations. In sharp contrast to the discoloration which was displayed so prominently in the irradiation products of sunlight, very little discoloration appeared during artificial irradiation. During the period of longest exposure to the lamp, the color did not progress beyond a faint yellow.

When the results of the same analytical procedure are compared for the two sources of radiation, the ultraviolet lamp was quantitatively more effective under the conditions of the experiments. The degree of decomposition which was found by turbidimetry after 48 hours exposure to the lamp through quartz was equivalent to that of at least 100 days of sunlight through glass. The effect of the composition of the containers for the irradiated solutions on photodecomposition is explained by a correlation of the spectral transmission of quinine (5, 13) and of soft glass, Pyrex, and quartz (3), since only the last transmits freely those wave-lengths which are absorbed by the alkaloid.

The results of other experiments on the irradiation of quinine by a mercury arc lamp are summarized in Table I. These results provide a comparison of the effect of a change in the concentration of quinine and in the

TABLE I

Irradiation of Quinine Sulfate and Quinine Dihydrochloride with Quartz Mercury Arc Lamp

	Analytical methods and results, apparent quinine				
	Turbidimetry of silicotungstates			Fluorometry	
Exposure	Direct, no extraction		Previous extraction	Direct	Extraction
	Hydrochloride	Sulfate	Hydrochloride	Sulfate	Hydrochloride
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	102.3	105.3		100.0	
12	54.2	54.8		100.0	
·24	51.0	45.0	56.4	96.6	
36	48.7	41.3	42.8	96.2	
48	46.2	37.2	48.1	94.7	96.0

composition of the acid which serves as the solvent. Both the hydrochloride and the sulfate which are recorded in Table I contained 6 gm. of the alkaloid as the free base in 2.00 N hydrochloric acid and 0.100 N sulfuric acid respectively. When compared turbidimetrically the hydrochloride solutions which are described both in Table I and in Fig. 2 decompose at approximately the same rate, although the concentration of quinine in the former is 3 times that of the latter. The same conclusion is reached if the sulfate and hydrochloride are compared. Little or no significant difference in the rate of decomposition of the sulfate and hydrochloride is shown in the data of Table I when the comparison is made on either a fluorometric or a turbidimetric basis, although the two different acids are used at extremely different concentrations. The two hydrochloride solutions which are compared above by turbidimetric analysis do show a difference in their rate of decomposition by a fluorometric comparison, the more concentrated solution showing less decomposition during a specified period of irradiation.

The irradiation of quinidine, 20 mg. per liter in 0.1 N sulfuric acid, was shown by fluorometric analysis to contain 60, 44, and 31 per cent of the original quinidine after 1, 2, and 3 hours exposure respectively to the mercury arc lamp. This is much more rapid than the decomposition of quinine in the same acid. This is attributed to the extreme difference in the concentration of the quinine and quinidine solutions, since the rate of photodecomposition of quinine is influenced by its concentration when the change is followed fluorometrically.

A chemical interpretation of the nature of the decomposition is difficult when one compares the results which were obtained by the fluorometric and turbidimetric procedures. Although the latter is the less specific of the two methods, its validity has been established for quinine in the absence of interfering substances (10). Therefore, this method indicates reliably the maximal but not the minimal amount of quinine *per se* which remains in the irradiated solutions, since some reaction product of quinine may also respond along with any quinine which may remain. The properties of the fluorescent cinchona alkaloids and their derivatives suggest that few structural changes can occur without the loss of fluorescence (7). The results impose, however, the conclusion that the reaction product of quinine retains the more specific property of fluorescence to a greater degree than it retains the less specific property of precipitation with an alkaloidal reagent. This conclusion does not include isomerization to quinotoxine which has no fluorescent properties and which does yield a silicotungstate turbidity.

Similar to our experience, Dietzel and Söllner (4) did not observe quantitative agreement between their methods of analysis of the solutions which they irradiated. They followed the reaction during irradiation by measuring the changes in the optical rotation and the boiling point elevation of the solution. As nearly as the conditions of their experiments can be correlated with those of ours, the changes in rotation are in general agreement. Their conclusion that a dimerization of the quinine molecule is brought about by ultraviolet irradiation is invalidated, however, by a recalculation of the data from their determination of boiling points. The decreasing effect of the solute on the elevation of the boiling point, which they determined at intervals of 3 hours during 12 hours exposure to a mercury lamp, is 4 times as great as the constant for water allows even if all solute particles had been removed from the original solution of quinine which they used.

No supposition can be offered from the evidence which is available at present that accurately describes the reaction which quinine undergoes during irradiation or the products which it yields.

SUMMARY

A quantitative study has been made of the photodecomposition of quinine, which occurs during its irradiation by sunlight and by an ultraviolet lamp under specified conditions.

Different analytical tools have been employed in following the photochemical reaction which quinine undergoes.

The influence of the concentration and the composition of the acid in which the alkaloid was dissolved during irradiation has been evaluated.

The photodecomposition of quinidine has been compared with that of quinine.

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IDENTIFICATION BY DISTRIBUTION STUDIES

VI. ISOLATION OF ANTIBIOTIC PRINCIPLES FROM ASPERGILLUS USTUS

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It was recently reported by Kurung (1) that the mold *Aspergillus ustus* produces a substance which inhibits *in vitro* the growth of both *Mycobacterium tuberculosis* and *Mycobacterium ranae*. Kurung further noted that the active principle could be extracted with ether and other organic solvents from the medium on which the mold was cultured. The problem of further purification and isolation of the active substance or substances present in the extract has been undertaken in this laboratory.

An excellent opportunity was thus offered to test the practicability of the use of the recently devised "counter-current distribution" technique (2, 3) for the isolation of biologically active principles from such crude preparations as the one at hand. The possibilities and particular advantages of this method in the isolation, characterization, and proof of homogeneity of an unknown substance, whether an active principle or otherwise, have been previously pointed out (2) and do not require repetition here. The initial experiments with the method in attacking the present problem met at once with sufficient success to warrant, in our estimation, publication of the results, even though they are at present only of preliminary character.

As set forth in the previous publications (2, 3), the only requisites for the application of the method are, first, that the substance must have such solubility relationships that it can be distributed between two immiscible liquids (or a liquid-solid system (4)) in equilibrium so that the effective partition coefficient is not too far removed from one, and, second, that a method of quantitative estimation, biological or chemical, must be available. Little difficulty was experienced in the present case of satisfying these requisites.

In the beginning, the amount of substance present was estimated by its antibiotic activity, but this was soon replaced for the more quantitative work by an extinction coefficient obtained with the Beckman ultraviolet quartz spectrograph at a favorable wave-length. The latter method of analysis was possible when a correlation between absorption spectrum and antibiotic activity had been established through the data obtained in a preliminary distribution. Cyclohexane and buffer were the two immiscible phases which gave a partition coefficient within a suitable range.

CHANGES THAT OCCUR IN PLASMA PROTEINS DURING GROWTH OF THE DOG

By LENA A. LEWIS

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Very few studies of change in plasma proteins incident to growth have been made. In 1918, Hatai (1) measured the changes with growth in the refractive index of rat serum, from which some information concerning the proteins could be drawn. Smith and coworkers (2, 3) studied total nitrogen in rat serum from the time the animals were weaned to full maturity, and albumin and globulin changes during the period of most rapid growth. The fractionation of the proteins was made by the Howe precipitation method. In order to obtain further information concerning the effect of growth on the different proteins, the present studies with the Tiselius electrophoresis technique were undertaken. Dogs were selected as the experimental animal so that the changes could be followed on the same subject throughout. By the electrophoresis technique with phosphate buffer, pH 7.8, six fractions are separated: albumin, α_1-globulin, α_2-globulin, β-globulin, γ-globulin, and fibrinogen.

Methods and Material

Four litter mates, mongrel puppies (two male and two female), were studied from the time of weaning at 6 weeks until full growth was attained. The diet consisted of cow's milk, Purina dog chow, and ground horse meat. When the animals were 4 months of age, the milk was discontinued. No attempt was made to determine the amount of food ingested by each dog. Blood was collected from the femoral artery approximately once a month. More frequent bleedings were avoided so that alterations could not be attributed to blood loss. Total protein nitrogen was determined by the Pregl modification of the micro-Kjeldahl method. A non-protein nitrogen of 25 mg. per 100 ml. was assumed. The Longsworth modification of the Tiselius electrophoresis technique was used to fractionate the proteins (4).

Results

The details of composition of the plasma of Dog 47 are shown in Fig. 1 to illustrate the trends observed. While the absolute values varied considerably from dog to dog, the general shift was similar in the four animals studied. The total plasma protein at the time of the initial examination fell between 3.6 and 4.4 gm. per 100 ml. There was no correlation between

IDENTIFICATION BY DISTRIBUTION STUDIES

VI. ISOLATION OF ANTIBIOTIC PRINCIPLES FROM ASPERGILLUS USTUS

By GEORGE H. HOGEBOOM and LYMAN C. CRAIG

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It was recently reported by Kurung (1) that the mold *Aspergillus ustus* produces a substance which inhibits *in vitro* the growth of both *Mycobacterium tuberculosis* and *Mycobacterium ranae*. Kurung further noted that the active principle could be extracted with ether and other organic solvents from the medium on which the mold was cultured. The problem of further purification and isolation of the active substance or substances present in the extract has been undertaken in this laboratory.

An excellent opportunity was thus offered to test the practicability of the use of the recently devised "counter-current distribution" technique (2, 3) for the isolation of biologically active principles from such crude preparations as the one at hand. The possibilities and particular advantages of this method in the isolation, characterization, and proof of homogeneity of an unknown substance, whether an active principle or otherwise, have been previously pointed out (2) and do not require repetition here. The initial experiments with the method in attacking the present problem met at once with sufficient success to warrant, in our estimation, publication of the results, even though they are at present only of preliminary character.

As set forth in the previous publications (2, 3), the only requisites for the application of the method are, first, that the substance must have such solubility relationships that it can be distributed between two immiscible liquids (or a liquid-solid system (4)) in equilibrium so that the effective partition coefficient is not too far removed from one, and, second, that a method of quantitative estimation, biological or chemical, must be available. Little difficulty was experienced in the present case of satisfying these requisites.

In the beginning, the amount of substance present was estimated by its antibiotic activity, but this was soon replaced for the more quantitative work by an extinction coefficient obtained with the Beckman ultraviolet quartz spectrograph at a favorable wave-length. The latter method of analysis was possible when a correlation between absorption spectrum and antibiotic activity had been established through the data obtained in a preliminary distribution. Cyclohexane and buffer were the two immiscible phases which gave a partition coefficient within a suitable range.

The mold[1] was cultured at 27–28° on shallow layers (about 200 ml.) of Czapex-Dox medium containing 4 per cent glucose and 0.1 per cent Bacto yeast extract. Between the 14th and 16th days after inoculation, each culture, including both the mold and the medium, was extracted twice with 2 volumes of ether, and the ether was removed by distillation *in vacuo* at room temperature. Approximately 150 mg. of yellow, gummy residue were obtained from each culture. The ether residue was dissolved in phosphate buffer in the range of pH 11. Acidification of this alkaline solution yielded a light yellow flocculent precipitate which was washed with water and dried *in vacuo*. The antibiotic activity of this crude residue, the acid precipitate and the fractions isolated from it by the counter-current distribution method, was estimated by the use of *Mycobacterium ranae*,[2] which was cultured at 38° in a thin film on 15 ml. of Long's liquid medium in small Blake bottles. This organism was found to be useful in preliminary tests because its rapid rate of growth permitted a result to be read in 24 hours.

The ether residue caused complete inhibition of the growth of *Mycobacterium ranae* at a dilution of 1:150,000 and perceptible inhibition at 1:750,000. Tests of the activity of the acid precipitate showed complete inhibition at a dilution of 1:300,000 and perceptible inhibition at 1:1,500,000. The filtrate remaining after separation of the acid precipitate contained no growth-inhibiting substances.
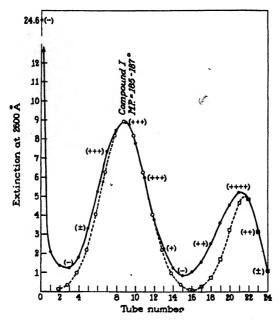
The acid precipitate was readily soluble in 0.1 N NaOH, ethanol, acetone ether, and benzol, somewhat less soluble in cyclohexane, and practically insoluble in water and 0.1 N HCl at room temperature. The material was rendered largely inactive by heating for 10 minutes at 100° in 0.1 N NaOH. The absorption curve of the material (0.40 mg. per ml. of ethanol) showed no definite bands but did show a plateau between 2500 and 2700 A with an extinction of 9.5. Preliminary tests showed that a distribution coefficient of 1, as determined by direct analysis at 2600 A, could be obtained when the acid precipitate was distributed between cyclohexane and 0.2 M $Na_4P_2O_7$ buffer containing 0.25 equivalent of H_2SO_4 (pH 8.31). An eight plate counter-current distribution analysis (3) with 9.6 mg. of acid precipitate and the above two phases demonstrated that the antibiotic activity closely followed the extinction observed at 2600 A in each of the cyclohexane layers and further indicated that there were at least two biologically active substances in the starting material.

A more detailed study of the acid precipitate was then carried out as

[1] The strain of *Aspergillus ustus* isolated by Mr. Kurung of the New York State Hospital for Incipient Pulmonary Tuberculosis, Ray Brook, New York, was kindly provided by him for the present experiments.

[2] American Type Culture Collection, No. 110.

follows: 50 mg. of the material, distributed between 8.0 ml. of cyclohexane and 8.0 ml. of the pyrophosphate buffer, were subjected to a twenty-four plate run in a machine similar to that described by Craig (2). After completion of the run, the material dissolved in the buffer of each tube was transferred to the cyclohexane layer by acidification and shaking. The amount of material in each tube was then estimated spectroscopically at 2600 A, the solution evaporated to dryness, and the residue tested for antibiotic activity. The results of this experiment are shown graphically

FIG. 1. Distribution of acid precipitate. ● = experimental values; ○ = calculated distribution for partition coefficient of 0.58; □ = calculated distribution for partition coefficient of 7.6; + and − refer to biological activity at equal dilution, *e.g.*, (++++) indicates complete inhibition of growth.

in Fig. 1. In this curve the extinction, a figure directly proportional to the weight or amount, is plotted against the consecutive number of the tube. As can be seen, the antibiotic activity coincided with the two peaks obtained by spectroscopic estimation. Moreover, the shape of the first band (Tubes 4 to 14) and its close agreement with that of the theoretical distribution of a pure substance with the same partition coefficient indicated the presence of a homogeneous compound. A crystalline residue was present in the tubes of this band after removal of the cyclohexane. When recrystallized from an ether-cyclohexane mixture and dried, this substance (Compound I) appeared in heavy square plates and melted at

185–187° (hot stage). The melting point did not change on recrystallization. On analysis, it was found not to contain N, S, or OCH₃ (C 53.37, H 3.64).[3] An approximate molecular weight of 350 was obtained by the Rast method in camphor, but this value must be taken with reservation, particularly because of the numerous oxygens as indicated from the carbon and hydrogen analysis. The substance was found to be an acid and gave a neutralization equivalent approximating 250 by direct titration in dilute alcohol against phenolphthalein with 0.1 N NaOH. The absorption spectrum was characteristic with a maximum at 3250 A and seemed to indicate an aromatic nucleus. The crystals showed approximately the same degree of antibiotic activity as did the acid precipitate, causing complete inhibition of the growth of *Mycobacterium ranae* at a dilution of 1:300,000 and perceptible inhibition at 1:1,500,000. The small amount of oily residue obtained from the mother liquor after recrystallization of Compound I was less than one-fourth as active as the pure substance.

The asymmetrical appearance and width of the second band in Fig. 1 (Tubes 16 to 24), when compared with the theoretical, indicated that more than one substance was present. Moreover, attempts to obtain crystalline material from these tubes without further distribution were unsuccessful, except in the case of Tube 18, which yielded approximately 0.5 mg. of crystals melting at 212–215°. The fact that the components of this band were caused to migrate slightly by the buffer but, in general, tended to remain in the cyclohexane layer, made it probable that a separation would be effected by a more alkaline buffer. Accordingly, further study of this mixture and, simultaneously, the isolation of the larger amount of Compound I were accomplished as follows: First, a twelve plate counter-current distribution was carried out with 160 mg. of acid precipitate, 30 ml. of cyclohexane, and 30 ml. of pyrophosphate buffer, pH 8.31. This run was made with separatory funnels (3). A total of 50 mg. of pure Compound I was isolated from the tubes which formed the symmetrical band. The remaining biologically active material, which migrated only slightly with the buffer and which corresponded to the asymmetrical band of Fig. 1, was collected. This latter material was then subjected to a machine run of twenty-four plates, utilizing the phases cyclohexane and 0.2 M Na₄P₂O₇ buffer at a pH of 8.66. The results of this analysis are shown in Fig. 2. 12 mg. of a crystalline substance, appearing in rosettes of fine needles, melting at 214–216°, and very similar to Compound I in solubility characteristics, were isolated from Tubes 10 to 19 of the symmetrical band (C 58.02, H 4.19).[3] The molecular weight obtained by the Rast method in

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[3] Since this paper was sent to press, surprising chlorine analyses of 22.63 and 16.84 for Compounds I and II, respectively, have been obtained. Formulations of $C_{21}H_{17}Cl_3O_6$ and $C_{21}H_{18}Cl_2O_6$ are thus suggested.

camphor approximated 330. A type of absorption spectrum similar to that of Compound I was found.

The second pure substance, Compound II, completely inhibited the growth of *Mycobacterium ranae* at a dilution of 1:100,000 and caused perceptible inhibition at 1:6,000,000. It thus appeared to be somewhat less active than the acid precipitate in inducing complete inhibition of growth,
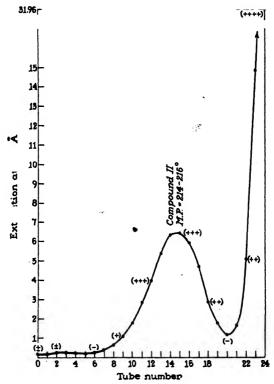
Fɪɢ. 2. Distribution of material corresponding to that present in Tubes 16 to 24 of Fig. 1.

but apparently caused slight but perceptible inhibition at dilutions beyond the activity range of the acid precipitate.

Tubes 22, 23, and 24 (Fig. 2) contained material only partially crystalline but possessing a considerable amount of antibiotic activity, a finding which demonstrated that in addition to Compounds I and II the original acid precipitate contained at least one other active substance. The results of a study of the third fraction and the further characterization of Compounds I and II, particularly in regard to their toxicity and effect on

Mycobacterium tuberculosis and other organisms, will be reported in the near future.

The present experiments thus demonstrate the practical value of counter-current distribution in this or any similar study to which the principle may be applied. Perhaps the most important advantages of the method are the following and are worth repeating here (2, 5, 6). (1) In empirical work, the method quickly gives a general analysis of the problem. This includes an estimate of the number of active principles or substances, their relative amounts, their differences in terms of partition coefficients, and the advisability of proceeding further along the same line. This information allows one to move logically from one step to the next with little waste of time or effort. (2) The method often effects sharp separation, usually in excellent yield of substances which are very similar in properties and otherwise difficult to separate. (3) It does not involve drastic chemical procedures which would be liable to result in the decomposition of labile material. In the present study the material was never subjected to a temperature higher than 40°. (4) It minimizes the chances of losing an important substance which is present in very small amount. (5) The symmetry or shape of an observed band provides criteria of homogeneity at the same time the fractionation is made. (6) The partition coefficients offer physical constants at once useful for identification and characterization.

It is our intention to apply the method of counter-current distribution to the isolation of other active principles of biological interest as opportunity presents itself. We also are further developing the theory and will attempt to devise practical improvements in the technique as well.

SUMMARY

1. Two crystalline antibiotics and a third partially crystalline active fraction have been isolated by means of "counter-current distribution" from a crude extract of the mold, *Aspergillus ustus*.

2. The comparative ease with which the fractionation was made demonstrates the usefulness of counter-current distribution in the examination of crude mixtures and for the final isolation and characterization of the substances or active principles present.

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THE EFFECT OF CERTAIN EXPERIMENTAL CONDITIONS ON THE FORMATION OF THYROXINE FROM DIIODOTYROSINE*

BY E. P. REINEKE AND C. W. TURNER

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The formation of thyroxine from diiodotyrosine when incubated for 2 weeks in a mildly alkaline solution at 37° was first reported by von Mutzenbecher (1). Subsequent investigators (2–4), using essentially the same method of incubation, obtained very similar results. Harington (5) stated that increased yields of thyroxine could be obtained at steam bath temperatures by the addition of hydrogen peroxide and constant shaking with *n*-butanol to extract the thyroxine as it is formed.

In previous reports from our laboratory, it has been shown that when sufficient iodine is combined with a protein to substitute 2 atoms per molecule of tyrosine (5) the amount of thyroxine formed can be influenced by the temperature of incubation (6, 7), the amount of agitation, and the presence of a catalyst (8). It seemed of interest, therefore, to determine whether the conditions established for the maximum formation of thyroxine in iodinated proteins would be operative when applied to diiodotyrosine.

EXPERIMENTAL

In most of the experiments, 21.6 gm. (0.05 M) of diiodotyrosine[1] were dissolved in 700 ml. of alkaline solution and incubated for 18 to 20 hours at the selected temperature. The solutions were stirred continuously by means of stirring motors adjusted to approximately 600 R.P.M.

Thyroxine Isolation Procedure—The thyroxine was isolated by a procedure similar to that described by von Mutzenbecher (1). In the earlier trials the reaction solution was diluted with 6 volumes of distilled water. Dilute sulfuric acid was added until the reaction became faintly acid to Congo red (pH 5.0), and the dark, flocculent precipitate was collected by centrifuging at once. A part of the unaltered diiodotyrosine usually crystallized out of the supernatant solution after standing for a few hours. In some of the later experiments, in which it was desired to recover the unchanged diiodotyrosine as completely as possible, the acid-insoluble material

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[1] We are indebted to Albert L. Elder, Director of Research, Corn Products Refining Company, Argo, Illinois, for generously supplying the diiodotyrosine used in this investigation.

was precipitated by adding dilute hydrochloric acid directly to the undiluted reaction mixture in an amount sufficient to redissolve the diiodotyrosine that crystallized at about pH 5.0. The acid-insoluble substance still remained precipitated and could be recovered by centrifuging. The diiodotyrosine was recovered from the supernatant solution by first adding sufficient saturated sodium hydroxide to make it slightly alkaline, and then acidifying with glacial acetic acid, whereupon the diiodotyrosine crystallized at once.

In either case, the acid-insoluble precipitate was dissolved in 300 ml. of 0.1 N sodium hydroxide solution, and then sufficient dilute hydrochloric acid was added to produce a definitely yellow color when the solution was tested with bromocresol green indicator.

The thyroxine was extracted by shaking the acidified solution in turn with 300 ml. and 150 ml. of n-butanol, and separating each time in a separatory funnel. The n-butanol extract was then shaken successively with 450, 225, and 100 ml. of 2 N sodium hydroxide solution to remove alkali-soluble impurities. The n-butanol extract was filtered, and the solvent was removed by vacuum distillation.

The residue remaining after removal of the n-butanol was dissolved in 100 ml. of 0.1 N sodium hydroxide. Then dilute acetic acid was added until a flocculent, light yellow precipitate formed. The precipitate was washed several times with 5 per cent acetic acid solution, and finally dissolved in a minimum of boiling sodium carbonate solution. A heavy white precipitate of the monosodium salt of thyroxine usually appeared immediately when the solution was cooled.

The solution was left in the refrigerator overnight, and then the monosodium thyroxine was recovered by centrifuging, dissolved in 70 per cent alkaline alcohol, and centrifuged again to remove the trace of undissolved material. When a few drops of glacial acetic acid were added to the boiling solution, thyroxine crystallized at once in the typical bundles of microscopic needles.

Factors Affecting Thyroxine Formation—In preliminary experiments conducted to establish, roughly, the most desirable medium for the conversion of diiodotyrosine to thyroxine, the results obtained with 1.0 N sodium hydroxide, 0.116 N sodium hydroxide, and 7 per cent sodium bicarbonate were compared. In all cases, 21.6 gm. of diiodotyrosine were dissolved in 700 ml. of solution and incubated at 70°, with vigorous stirring, for 20 hours. Under these conditions diiodotyrosine was highly stable in 1.0 N sodium hydroxide, only traces of acid-insoluble material being formed. Crystalline thyroxine was recovered after incubation in both the 7 per cent sodium bicarbonate and 0.116 N sodium hydroxide solutions. Considerably more oxidative side reactions appeared to occur in the sodium bicarbonate than in

the sodium hydroxide medium, since the acid-insoluble precipitate obtained was darker, and a smaller yield of thyroxine was obtained. Therefore, all of the subsequent preparations were incubated in 0.116 N sodium hydroxide solution. Under the conditions used the solutions remained at a pH of approximately 9.4 to 9.6 throughout the process.

Effect of Catalyst and Incubation Temperature—From our previous results with iodinated proteins (7, 8), it was expected that the incubation temperature would have an influence on the amount of thyroxine formed under the given conditions. It also seemed of interest to determine whether manga-
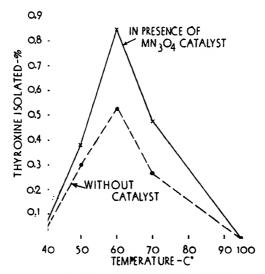
Fig. 1. Gross yield of thyroxine isolated after incubation of diiodotyrosine at various temperatures.

nese oxide (Mn_3O_4) would exert the same catalytic effect with diiodotyrosine as was observed with iodinated casein (9).

In order to answer these questions, diiodotyrosine solutions were incubated at various temperatures in the range of 40–94°, and subjected to the isolation procedure described. At each temperature interval one sample was incubated in 0.116 N sodium hydroxide alone; to a second sample, otherwise treated identically with the first, 2 gm. of manganese oxide were added.

At 40° (Fig. 1), only a trace of thyroxine was formed, amounting to gross yields of 0.03 and 0.04 per cent. With both types of treatment, thyroxine formation increased rapidly with increasing temperature until the optimum

of 60° was reached. There was a decline in the amount of thyroxine recovered at 70°, with zero recovery at 94°. Throughout the effective temperature range there was an increased recovery of thyroxine from the samples incubated in the presence of manganese oxide, confirming the results obtained previously with iodinated casein (9).

In solutions incubated at 94° large amounts of dark colored acid-insoluble material were formed, despite the fact that no thyroxine could be recovered. This suggested the possibility that thyroxine formed early in the process might have been altered by further oxidation, and it seemed possible that if this were true the thyroxine could be protected by conducting the process in the presence of n-butanol (5, 10). No thyroxine could be recovered, however, from solutions incubated at 94° with an equal volume of n-butanol added.

TABLE 1

Effect of Stirring and Aeration on Formation of Thyroxine from Diiodotyrosine

Incubation temperature	Thyroxine yield	Treatment
°C.	*per cent*	
40	0.04	Mn_3O_4, 2 gm.; stirred at 600 R.P.M.
40	0.04	" 2 " aerated vigorously
50	0.38	" 2 " stirred at 600 R.P.M.
50	0.36	" 2 " aerated vigorously
60	0.85	" 2 " stirred at 600 R.P.M.
60	0.52	No catalyst; " " 600 "
60	0.02	Mn_3O_4, 2 gm.; no stirring or aeration
70	0.27	No catalyst; stirred at 600 R.P.M.
70	0.01	" " no stirring or aeration

The maximum yield of thyroxine obtained at the optimum incubation temperature of 60° was 183 mg., equivalent to a gross yield of 0.85 per cent. However, of the diiodotyrosine initially taken all but 6.5 gm., on the average, can be recovered after separating the thyroxine. Based on this figure, the net yield of thyroxine is 2.8 per cent.

In addition to the points illustrated in Fig. 1, the incubation system employed provided an opportunity to study the effect of such factors as the amount of stirring and aeration on the process of thyroxine formation. Some preparations were aerated continuously during incubation by bubbling finely dispersed air through them. Both the stirring and aeration were omitted in others, although the surface of the solutions was still in contact with the atmosphere.

With other conditions being held constant, practically identical yields of thyroxine were obtained whether the solutions were stirred vigorously or

aerated (Table I). With the stirring and aeration omitted, only traces of thyroxine were recovered. This was true even when the usual amount of catalyst was added. Thus it is evident that the formation of thyroxine can be brought about by atmospheric oxygen. Manganese oxides will catalyze this reaction, but are ineffective in the absence of oxygen.

Identity of Thyroxine Obtained—A total of 2.012 gm. of thyroxine obtained in these experiments was pooled and recrystallized twice from sodium carbonate solution. The pure white monosodium salt was then dissolved in 70 per cent alkaline alcohol, and free thyroxine was crystallized from the boiling solution by the addition of acetic acid. Thyroxine appeared at once as the typical bundles of microscopic needles, in a yield of 1.416 gm. The melting point, when heated at the rate of 3° per minute, was 230–231°. Analysis showed 65.2 per cent iodine, compared to the theoretical of 65.4 per cent.

Harington (5) stated that the thyroxine formed from natural tyrosine in the presence of *n*-butanol was levorotatory. Polariscopic examination showed the thyroxine obtained in the present instance to be a racemic mixture, even though optically active diiodotyrosine was used as starting material. Racemization apparently had taken place during the incubation in alkaline solution at an elevated temperature.

DISCUSSION

It is of considerable interest to note the similarity between the conditions required for the maximum conversion of diiodotyrosine to thyroxine, whether it is combined in an iodinated protein or in the free state.

In a previous report (7), it was shown that a pronounced increase in the thyroidal activity of iodinated protein is obtained by incubation at 60–70°. The present investigation shows that a definite temperature optimum for the formation of thyroxine from diiodotyrosine occurs at 60°. Incubation at 40°, the temperature commonly employed (1–4), yields only a trace of thyroxine in a 20 hour period.

Likewise, this reaction is catalyzed by manganese oxide in iodinated proteins (9) and in diiodotyrosine, as shown in the present report.

It was reported by Barkdoll and Ross (4) that diiodotyrosine incubated in an oxygen-free atmosphere does not form thyroxine. Conversely, the yield of thyroxine was increased by bubbling a slow stream of air through the solution. The importance of atmospheric oxygen is also evident from the present work, since only traces of thyroxine are formed unless air is incorporated in the solution, either by stirring or direct aeration. This is true even at the optimum temperature and in the presence of a catalyst.

Von Mutzenbecher (1) originally suggested that the formation of thyroxine in iodinated proteins could be brought about by the oxidative cou-

pling of 2 molecules of diiodotyrosine, with the elimination of one side chain. A detailed mechanism for this reaction was worked out by Johnson and Tewkesbury (3), and elaborated further by Harington (5). It was suggested by all of these workers that the oxidation is brought about by the action of hypoiodite. If this were the case, however, thyroxine formation should continue even in the absence of additional oxygen. From the results presented in Table I it is obvious that, under the conditions used, aeration of the solutions is essential for the formation of appreciable amounts of thyroxine.

From the fact that manganese will catalyze thyroxine formation only in the presence of oxygen it appears to act as an oxygen carrier for the oxidative coupling reaction that is apparently involved.

As pointed out previously (9), demonstration of the catalysis of thyroxine formation by manganese *in vitro*, together with the special capacity of the thyroid for the storage of manganese, suggests the probability that this element also plays a part in promoting the synthesis of thyroxine in the thyroid gland.

SUMMARY

Diiodotyrosine dissolved in 0.116 N sodium hydroxide (pH 9.4 to 9.6) was incubated with vigorous stirring for 18 to 20 hours under various conditions, and the amount of thyroxine formed was determined by isolation as the crystalline product.

It was found that a definite temperature optimum for the formation of thyroxine under these conditions occurs at 60°.

Thyroxine formation during the incubation is greatly accelerated by atmospheric oxygen, whether incorporated by stirring or by direct aeration.

Only traces of thyroxine are formed in solutions that are neither stirred nor aerated. Manganese oxide was shown to catalyze the reaction in the presence of atmospheric oxygen.

With all of the known conditions at the optimum, a yield of thyroxine equivalent to 0.85 per cent of the diiodotyrosine taken initially was obtained. If allowance is made for the recovery of unaltered diiodotyrosine, this is equivalent to a net yield of 2.8 per cent. The identity of the thyroxine was verified by its iodine content of 65.2 per cent and *melting point of 230–231°*. The thyroxine was optically inactive, apparently because of racemization under the conditions of incubation employed.

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STUDIES IN CARBOHYDRATE METABOLISM

VI. EFFECTS OF HYPO- AND HYPERINSULINISM IN RABBITS*

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In previous studies of rats rendered diabetic with alloxan (1, 2) evidence was presented for the impairment of utilization of glucose in these animals, not only in the process of glycogenesis but also in lipogenesis. Whereas the finding of stably bound deuterium in glycogen of both liver and muscle, when the body fluid was enriched with D_2O, proved that glycogenesis had not ceased, the data indicated that the glycogen that was formed arose predominantly from small fragments, such as lactate, rather than from hexose directly. The fatty acids isolated from the liver fat of these animals were extraordinarily low in deuterium concentration, showing that the normally important route of glucose utilization, hepatic lipogenesis, had almost come to a standstill. It could be estimated that the diabetic rats formed new molecules of fatty acid at a rate approximately 5 per cent of normal.

These findings led to an attempt to determine whether the converse effect, abnormally large utilization of glucose in these processes, occurs when insulin is injected into otherwise normal animals. This question was partially answered when it was shown that in the normal rat treated with insulin and deuterium oxide the bulk of the glycogen deposited in the muscle arose directly from hexose. The glycogen isolated from this source, though greatly increased in quantity, was poor in isotope, a finding which demonstrated that the dietary glucose was incorporated into glycogen fairly directly. It was pointed out (2) that an increase in the rate of lipogenesis in the liver might or might not result in an increase in the quantity of liver fat, depending upon the integrity of the fat transport systems, but that, with the body fluids enriched with heavy water, it would necessarily result in a rise in the deuterium concentration of the liver fatty acids. Such an increase we have failed to demonstrate in the rat to date.

An objection to the use of rats in this type of experiment is that the rat, when fed *ad libitum*, is remarkably resistant to insulin shock. We have given as much as 60 units of insulin per day to a rat without inducing symptoms of shock. Because of this peculiarity of the rat, we have elected to

use rabbits in the experiments to be described.   At least in this regard, the response of the rabbit is more like that of the human.

The D_2O concentration of the body fluids was raised and maintained at a suitable concentration (3) for a period of 48 hours.   During this period the rabbits were fed *ad libitum* a high carbohydrate, low fat ration.   Fatty acids from liver and depot, and glycogen from liver and muscle, were isolated and their deuterium content compared with that of the body water (Table I).   By this procedure four rabbits have been studied, two previously rendered diabetic by the intravenous injection of alloxan, one normal, and one normal rabbit that was given hypodermically each day slightly less than the shock-producing dose of zinc protamine insulin.

TABLE I

Deuterium Concentrations in Fatty Acids and Glycogen from Rabbits

Weights of liver constituents are reported as per cent of the wet liver weight, weights of carcass constituents as per cent of body weight.   Deuterium concentrations are reported (a) as atom per cent excess, determined analytically, and (b) as per cent of the deuterium concentration in the body water of the corresponding animal.

Rabbits	Body water D (a)	Liver fatty acids			Carcass fatty acids			Liver glycogen			Carcass glycogen		
		Weight	D (a)	D (b)	Weight	D (a)	D (b)	Weight	D (a)	D (b)	Weight	D (a)	D (b)
Diabetic, No. 1	1.23	1.8	0.033	2.7	1.8	0.015	1.2	0.6	0.068	5.5	0.1	0.055	4.5
" " 2	1.18	4.5	0.016	1.4	1.0	0.013	1.1	0.4	0.067	5.7	0.1	0.038	3.2
Normal	1.12	1.9	0.074	6.6	3.7	0.009	0.8	2.9	0.320	28.6	0.1	0.052	4.6
" + insulin	1.30	1.9	0.339	26.1	5.0	0.030	2.3	5.9	0.112	8.6	0.2	0.048	3.7

In the selection of rabbits suitable for the administration of alloxan, it was found necessary to eliminate those suffering from coccidiosis.   Rabbits suffering from this disease, when given alloxan, go into a rapid decline, with marked anorexia and cachexia, leading to death in about 2 weeks. Rabbits with stool smears negative for coccidia were found to tolerate alloxan well.

Of the two animals selected for the isotope experiments, the second was more severely diabetic than the first, as judged by the glucosuria.   However, except for the mild fatty liver exhibited by the second rabbit, the two animals yielded similar analytical results.   Precisely as previously reported in rats (1, 2), in diabetic rabbits the depot fat is diminished in quantity and the liver fatty acids are markedly poorer in deuterium than in the normal animal.   This we believe to reflect a definite retardation in hepatic lipogenesis, a failure on the part of the diabetic animal to utilize glucose adequately for the synthesis of fatty acids.

The diabetic rabbits had subnormal quantities of glycogen in their livers. In contrast to the finding in the diabetic rat (1), this glycogen was notably poor in isotope. The probable meaning of this finding is that these rabbits formed but little glycogen from any precursor whatever, whereas the rat suffering from diabetes apparently continues to form glycogen from small fragments, even though it is incapable of directly utilizing hexose for this process at a normal rate.

When insulin was administered to a normal rabbit, the quantity of glycogen both in the liver and in the carcass increased. The deuterium concentration of this glycogen, however, was quite low, indicating that the extra glycogen formed in response to the administered insulin was made by a process that did not involve uptake of much hydrogen from the body water. For reasons previously outlined (4), we believe that this process is one which more or less directly utilizes the hexose of the diet, since glycogen formed from smaller fragments such as lactate is necessarily rich in isotope, under these experimental conditions. We have previously reported that in the rat receiving insulin the extra muscle glycogen that is deposited is likewise formed more or less directly from hexose (2).

The most striking finding in the rabbit that had been treated with insulin was the tremendous rise above normal in the deuterium concentration of the liver fatty acids. About 4 times as much deuterium had found its way into the fatty acids of the liver fat in 2 days in this animal as in the normal, untreated animal. This must mean that, as a result of the injection of insulin, the rate of hepatic lipogenesis had increased, probably at least 4-fold. The fact that no fatty liver developed simply means that the newly formed fat was being carried away from the liver efficiently, and the likelihood that some of it was transported to the depot is suggested by the apparent increase in both quantity and deuterium concentration of the carcass fatty acids.

These findings taken together warrant the conclusion that animals suffering from hypoinsulinism make fatty acids from carbohydrate precursors excessively slowly, while the administration of extra insulin results in excessively rapid hepatic lipogenesis. They should not be construed, however, to mean that insulin necessarily plays some specific rôle in lipogenesis. Rather it appears probable that the insulin level affects the rate of utilization of glucose in all of its manifestations and that lipogenesis is simply one of the several processes in which products derived from glucose are utilized. It seems likely that the interesting finding recently reported by Price, Cori, and Colowick (5) will aid in the interpretation of the present results.

The impairment of the diabetic rat's ability to form new fatty acids is probably related to the finding that such animals will, if offered the option, elect to eat a diet rich in fat and poor in carbohydrate (6) and, if allowed to do so, will thrive on such a diet (7). It is also possible that our finding that

the administration of insulin favors lipogenesis and subsequent deposition of the newly synthesized fat is related to the clinical use of insulin to stimulate gain in weight in underweight individuals (8).

The glucose appearing in the urine of the diabetic rabbits contained deuterium. The analogous finding with diabetic rats has been discussed in a previous communication (1, 2) at which time we gave our reasons for the belief that the appearance of isotope in urinary glucose represented synthesis of glucose from smaller fragments. In Table II are given the values of the deuterium concentrations in the daily urine glucose samples obtained from Diabetic Rabbit 2, and also values, calculated therefrom, for the per cent of the urinary glucose that was synthesized *in vivo*. It should be pointed out that in this calculation the simplifying though unproved as-

TABLE II

Deuterium Concentration in Urinary Glucose

Glucose has been isolated as the pentaacetate from each day's urine of Diabetic Rabbit 2, and its deuterium concentration compared with that of the simultaneously excreted water.

Time	Urine water	Urine glucose		Glucose synthesized
		Weight	D	
	(a)		(b)	$(c)^*$
hrs.	*atom per cent D*	*gm.*	*atom per cent*	*per cent*
0–24	1.05	52.2	0.264	43
24–48	1.09	54.0	0.304	48

$$\frac{100 \times 12 \times b}{7a}$$

sumption is made that the glucose of the urine is composed of two species, one of which is derived from the diet and devoid of stably bound isotope, and the other so synthesized in the animal that its carbon-bound hydrogen is at the same isotopic level as the hydrogen of the body water.

Granted this assumption, it would appear as though almost half of the urinary glucose was synthesized *in vivo* and about half was derived directly from the diet. The explanation provisionally offered to account for this finding is that the diabetic rabbit daily generated from small fragments a quantity of glucose almost as large as that ingested, mixed the two species of glucose, and excreted a portion of the resultant mixture in the urine. As the rabbit derived approximately 100 gm. of glucose from the diet daily, the quantity synthesized daily may be estimated as lying in this neighborhood. The proportion of synthesized to ingested glucose in the rabbit is somewhat larger than the corresponding value similarly estimated for rats, in which it

appeared that from one-third to one-half as much glucose[1] was synthesized each day as was eaten.

EXPERIMENTAL

Young female rabbits weighing between 1.8 and 2.4 kilos were kept in individual metabolism cages and offered food and water *ad libitum*. Rockland rabbit ration, stated to contain 61.66 per cent of carbohydrate and 2.65 per cent of fat, was used throughout.

Diabetes was induced by the intravenous injection of alloxan monohydrate, 150 to 200 mg. per kilo of body weight, in a single dose. To control the initial hypoglycemia, for a period of 24 hours following injection the drinking water was replaced by 5 per cent glucose solution. Because of several fatalities about 2 weeks after such treatment, in which the cause of death was assigned to dissemination of coccidiosis throughout the liver,[2] only rabbits which had negative stool smears were employed.

Diabetic Rabbit 1 was observed for a period of 1 month after administration of alloxan. At first, an occasional trace of acetone was found in the urine, later, none. 30 to 40 gm. of glucose were excreted daily in a urine volume of 600 to 700 cc. The food consumption averaged 175 to 225 gm. daily, and there was no marked weight loss.

Diabetic Rabbit 2 never exhibited ketonuria. During the week of preliminary observation it lost about 10 per cent of body weight, while consuming about 150 to 200 gm. of food daily. The daily urine volumes were 500 to 600 cc. and contained 36 to 54 gm. of glucose.

Fatal hypoglycemia was produced in a rabbit by the subcutaneous injection of 6 units of zinc protamine insulin. The rabbit employed in the isotope experiment was therefore given 4 units on the 1st day and 5 units on the 2nd day of the experiments. Doubtless because of the continuous accessibility of food supplies, this rabbit did not develop any marked hypoglycemia that was detected.

Enrichment of the body fluids with respect to D_2O was accomplished by the intravenous injection of 99.5 per cent D_2O, containing 0.9 per cent of NaCl, 1 cc. per 100 gm. of body weight. Simultaneously the drinking water was replaced by diluted D_2O, the concentration varying from animal to animal between 1.56 and 1.70 per cent, as estimated from preliminary observations of water and food consumption.

The rabbits were killed by the intravenous injection of air, the bodies eviscerated, and the carcasses rapidly dismembered and submerged in

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[1] These values were erroneously reported as lying between one-fourth and one-third in the previous paper (2).

[2] The authors are indebted to Dr. H. P. Smith and Dr. H. N. Eisner of the Department of Pathology, Columbia University, for the establishment of this diagnosis.

boiling 30 per cent KOH. The livers were also digested with hot alkali. The gastrointestinal tracts were discarded and samples of body water were distilled from the pooled remaining organs. Isolation and analytical procedures were the same as those previously described (9).

Samples of urine were distilled to obtain urine water. The remainder of the urine from the diabetic animals was treated with acetic anhydride and sodium acetate, as previously described (1), and glucose pentaacetate ultimately isolated, purified by recrystallization from hot water, and analyzed for deuterium.

SUMMARY

In the alloxan-diabetic rabbit, as previously shown to be true for the alloxan-diabetic rat, the rate of lipogenesis is well below normal. This is interpreted as a specific example of the more general impairment of glucose utilization in this condition.

The glycogen deposited in muscle and in liver in the rabbit in response to insulin has been shown to arise chiefly by fairly direct processes from dietary glucose.

When insulin is administered to a normal rabbit, a large increase in the rate of hepatic lipogenesis has been observed.

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SOME CORRELATIONS OF GROWTH-PROMOTING POWERS OF PROTEINS WITH THEIR STREPOGENIN CONTENT

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It is now generally believed that the nutritional or growth-promoting powers of proteins are reflected entirely by their content of the various amino acids. This view has arisen despite the fact that rats, mice, and chickens grow more rapidly when fed casein than when given a mixture of amino acids[1] free of protein (2–4). Opinions have varied as to why intact proteins show this nutritional superiority over mixtures of amino acids. Some have held that the improved growth is merely a reflection of differences in palatability. Therefore, the following new facts are presented, for they have led to the hypothesis that the nutritional superiority of some proteins is due to the presence in them of a specific factor which appears to be correlated with the occurrence of strepogenin.

Strepogenin is the tentative name used to designate a factor (or factors) which stimulates the growth of *Lactobacillus casei*, some hemolytic streptococci, and certain other microorganisms (5, 6). It has been found as an integral part of certain highly purified proteins (7, 8), from which it is liberated by tryptic digestion. Indeed, proteins have proved to be by far the richest sources of the growth factor. The active agent is destroyed by hydrolysis with strong acid or alkali. Because of these and other properties of the factor, it has been concluded that strepogenin is a peptide or peptide-like substance (7).

In view of the occurrence of strepogenin in certain proteins, it seemed possible that the superior growth-promoting properties of these substances might be associated with this factor. The idea was examined experimentally in several ways. Firstly, the amino acids derived from casein by acid hydrolysis, plus tryptophane and cystine, were substituted for protein in a highly purified diet which was fed to mice. The diet was free of strepogenin, for that present in casein was destroyed by the acid hydrolysis. Growth was considerably slower than that observed with similar diets containing intact casein. When casein or some other protein rich in strepogenin was added, a good rate of growth was obtained, while with proteins

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*With the technical assistance of M. L. Collyer. A preliminary report of some of the results has appeared (1).

[1] The fact that animals fed mixtures of pure amino acids actually do grow has tended to obscure the suboptimal rate of gain on such a nitrogen source.

low in or devoid of strepogenin, the rate of growth was not increased. The growth-promoting powers of the various proteins examined were directly correlated with their strepogenin contents. As little as 0.5 per cent of an active protein such as crystalline trypsinogen was sufficient to elicit maximal response, and the addition of 2 per cent vitamin-free casein gave similar results. Secondly, concentrates of strepogenin made from tryptic digests of casein were as active as the protein when fed on an equivalent strepogenin basis. Destruction of the strepogenin in these preparations by acid hydrolysis was attended by loss of growth-promoting effect in mice. Finally, a strepogenin-poor, but otherwise nutritionally complete protein (egg white) was used as a source of nitrogen in a highly purified basal diet instead of the mixture of amino acids. Growth was poor on such a diet except when a strepogenin-rich protein such as casein or trypsinogen was added. A concentrate of the growth factor prepared from tryptic digests of casein was also effective with this diet. Proteins low in strepogenin were without benefit.

While the correlation of growth-promoting activity for the mouse with strepogenin content seemed good, it cannot be said that the effect on mice was due to strepogenin. Before this can be done it will be necessary to obtain strepogenin in pure form and to test its potency. The available data show merely that there is a growth-stimulating material in some proteins which seems to parallel the content of strepogenin. Since this effect cannot be attributed to the known amino acids, it would seem that there is something of nutritional importance other than these amino acids present in certain proteins.

A deficiency of the growth-stimulating substance in certain proteins did not lead to complete failure of growth or to death of the animals. It merely resulted in diminished rate of growth. All the mice fed the basal diets eventually reached mature size. The only external sign other than poor growth was loss of hair on the top of the head which occurred in a few of the mice fed the basal diets.

<center>EXPERIMENTAL</center>

Methods of Assay—Weanling mice, ranging in weight from 10 to 12 gm., were caged individually on screen floors and fed the various rations described below *ad libitum*. Supplements were incorporated directly into the rations. Whenever crystalline trypsinogen was used as a supplement, fresh ration was prepared every 3 days because this protein was added as an aqueous suspension after heating, and in such a preparation strepogenin potency disappeared rather rapidly. The mice were weighed twice weekly over a 2 to 3 week period.

Strepogenin assays were carried out with *Lactobacillus casei*, according to the method of Sprince and Woolley described earlier (8).

Strepogenin Concentrates—Several strepogenin concentrates all prepared from tryptic digests of vitamin-free casein have been used. The first was made by the phenol extraction of the non-dialyzable portion of such a digest according to the method used previously (8). This was designated Concentrate A. Concentrate B was made from Concentrate A by adding an excess of picric acid to a 10 per cent neutral aqueous solution of the concentrate, cooling, filtering, and removing the picric acid from the filtrate by acidifying with HCl and shaking with ethyl acetate. The aqueous solution was then neutralized with NaOH and concentrated under reduced pressure to dryness. The residue was extracted with methanol, and the soluble portion was freed of solvent and extracted with ethanol. The resulting insoluble part was used.

Casein Hydrolysate—Vitamin-free casein was suspended in 10 times its weight of 7 N H_2SO_4 and heated in an autoclave at 20 pounds pressure of steam for 16 hours. Sulfate was exactly removed as $BaSO_4$, and the filtrate and washings from the latter were concentrated to dryness at low temperature. The mixture of amino acids was then dried in a vacuum and finely powdered. The material so obtained was rather hygroscopic.

Dialyzed, Coagulated Egg White—Fresh egg white was poured into shallow pans and heated in an autoclave until firm. The coagulum was ground, suspended in water, and the suspension was dialyzed against running water overnight. The dialysis was performed in cellophane tubes (sausage casings) and was necessary in order to remove the small amounts of free strepogenin contained in egg white. The heating was necessary to inactivate antibiotin (avidin). The dialyzed coagulum was filtered off on cheese-cloth, washed with alcohol or acetone, and dried in a current of air. When thoroughly dry, it was powdered in a ball mill.

Potency of Proteins and Concentrates When Added to Casein Hydrolysate Basal Rations—Two basal rations, which varied in the nature of the carbohydrate, were used. The first consisted of sucrose 750 gm., salts (9) 50 gm., casein hydrolysate 180 gm., *dl*-tryptophane 8 gm., *l*-cystine 3 gm., thiamine 2 mg., riboflavin 5 mg., nicotinic acid 10 mg., pyridoxine 2 mg., calcium pantothenate 20 mg., choline chloride 100 mg., inositol 1 gm., corn oil fortified with vitamins A, D, E, and K (10) 10 gm., and cellulose (Cellu flour) 100 gm. The cellulose was added in order to reduce the hygroscopicity of the ration. The tendency of mixtures compounded with casein hydrolysate to become sticky when exposed to air was overcome in the second ration by the use of heated starch instead of sucrose. The second ration therefore was the same as the first, except that the cellulose was omitted and the sucrose was replaced with amigel, a commercially available, heated corn-starch.[2]

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[2] Obtained from the Corn Products Refining Company.

The growth responses of mice fed either of these two diets alone or in combination with various proteins and strepogenin concentrates are shown by the data in Table I. The strepogenin contents of the several diets as measured by the *Lactobacillus casei* assay are also shown. It can be seen

TABLE I

Growth-Promoting Effects of Proteins and Concentrates Added to Diet Prepared from Casein Hydrolysate

Addition to basal ration	Strepogenin content of ration*	With sucrose basal		With starch basal	
		No. of animals	Average change in weight	No. of animals	Average change in weight
	per cent		*gm. per wk.*		*gm. per wk.*
None		0	12	+1.7	12	+3.4
Casein (vitamin-free)	10	0.5	4	+3.3		
	·5	0.25			9	+5.7
	2	0.10	4	+3.1		
	1	0.05	7	+2.4	3	+4.5
	0.5	0.025			4	+2.8
Heated crystalline tryp-sinogen	0.5	0.15	4	+3.6	8	+5.5
Native crystalline tryp-sinogen	0.5		4	−0.1		
Heated horse hemoglobin†	5	0.015			4	+4.0
	2	0.006			4	+3.9
Gelatin	5	0			4	+1.0
Dialyzed heated egg white	5	0.015	7	+2.1	5	+3.9
Whole dried beef liver	2	0.02			4	+5.1
Strepogenin, Concentrate A	Equivalent to 2% casein	0.10	8	+3.0	4	+4.8
Strepogenin, Concentrate B	" "	0.10			4	+6.0
Acid hydrolyzed, Concentrate A	Equivalent to 4% casein	0	4	+1.3		
Glutamine	2				4	+3.5

* The strepogenin values are relative to a standard liver extract which was assigned a potency of 1 (*cf*. (8)).

† A solution of dialyzed crystalline horse hemoglobin which had been coagulated in boiling water and dried was used. This preparation was lower in strepogenin than the hemoglobin examined previously (8).

that proteins such as casein and trypsinogen which were rich in strepogenin promoted growth, while those which were poor in that factor did not. The potency of liver in this test seemed correlated with its strepogenin content. Similarly the activity of concentrates prepared from tryptic

digests of casein was roughly proportional to their strepogenin contents, as far as could be judged by these results. With the proteins and concentrates tested, the correlation of activity for the mice with strepogenin content was not quantitative, but in view of the inadequacies of both the animal and bacterial assays involved, the divergence was not great.

Although the crystalline trypsinogen was very active after it had been denatured by heat, the native protein was rather harmful to the mice. This toxic effect of native trypsinogen likewise was observed in the trials with the egg white basal ration described below. The heated trypsinogen was prepared by dialyzing crystalline trypsinogen[3] and heating the aqueous solution of the protein in a boiling water bath for 5 minutes.

TABLE II

Growth-Promoting Effects of Proteins Added to Diet Prepared with Egg White

Addition to basal ration		No. of animals	Average change in weight
	per cent		*gm. per wk.*
None		18	+2.5
Casein (vitamin-free)	5	15	+3.7
	1	4	+3.2
Gelatin	5	6	+2.2
Dialyzed heated egg white	5	5	+2.2
Heated crystalline trypsinogen	0.5	5	+5.1
Native " "	0.5	6	−2.8
Strepogenin, Concentrate A	Equivalent to 2% casein	6	+4.0

Glutamine was tested because, under certain experimental conditions (11), it had strepogenin activity for *Lactobacillus casei*. However, it was inactive for the mice, just as it was for hemolytic streptococci which required strepogenin (5).

Growth-Promoting Properties of Heated Starch—The data in Table I show that the heated starch (amigel) used in the second ration had a marked growth-stimulating effect when compared to sucrose. This phenomenon was quite independent of and incidental to the enhancement of growth due to proteins. The animals receiving amigel and an active protein such as casein grew at a rate equal to or exceeding that seen in mice fed stock rations. In view of the fact that stock rations contain starchy materials, this growth-stimulating action of starch may explain the observation in several laboratories that mice grow slightly better when fed a stock ration than when given a purified diet compounded with

[3] The crystalline trypsinogen was very kindly supplied by Dr. M. Kunitz of The Rockefeller Institute for Medical Research.

glucose or sucrose. However, it may be that the superiority of the amigel was due to some unknown stimulant other than starch.

Growth-Stimulating Powers of Various Proteins Added to Ration in Which Egg White Was Source of Nitrogen—Since egg white was very low in strepogenin, and yet well supplied with the known amino acids, a ration could be prepared which was low in strepogenin but which contained unhydrolyzed protein as the source of nitrogen. This ration contained sucrose 750 gm., salts (9) 50 gm., dialyzed, heated egg white 180 gm., and the same quantities of vitamins as in the casein hydrolysate rations. The responses of mice to this mixture alone, and with the addition of various proteins, are shown in Table II. It can be seen that growth was poor on the basal ration, and that it was improved by the addition of a protein rich in strepogenin. Proteins low in strepogenin were not effective in this respect.

SUMMARY

When mice were fed a highly purified ration in which casein hydrolysate plus tryptophane and cystine was the nitrogen source, they grew at a submaximal rate. Small amounts of proteins rich in strepogenin were effective growth promoters, while proteins low in this peptide-like growth factor were not. Concentrates of strepogenin prepared from tryptic digests of casein were as active as the intact protein. When the strepogenin was destroyed in such concentrates, the growth-promoting powers for mice were lost. When egg white, a protein low in strepogenin, but otherwise nutritionally adequate, was used as the source of nitrogenous matter in a highly purified ration, a slow rate of growth was observed, and this situation was remedied by administration of small amounts of proteins rich in strepogenin, but not by those poor in this factor. Some implications of these findings for current theories of protein nutrition have been indicated.

A stimulation of growth caused by the addition of heated starch to highly purified rations has been described.

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LETTERS TO THE EDITORS

THE AVAILABILITY OF VITAMIN B₆ IN YEAST TO SACCHAROMYCES CARLSBERGENSIS*

Sirs:

In exploring the problem of the complete extraction of vitamin B_6 from natural sources, Melnick *et al.*[1] reached the conclusion that, in addition

Efficiency of Various Extraction and Hydrolytic Procedures in Rendering B_6 in Yeast 200-B Available to Saccharomyces carlsbergensis

Experiment No.	Extraction	"Hydrolytic" procedure*	B₆ found†	
			Steamed‡	Filtered§
			γ per gm.	γ per gm.
1	Digestion with clarase‖	None	36	39
2	" " "	2 N H₂SO₄		40
3	0.055 N H₂SO₄*	None	25	26
4	0.055 " "	2 N H₂SO₄	28	33
5	2 N H₂SO₄*	None	16	17
6	2 " "	2 N H₂SO₄	19	
7	2 " "	0.055 N H₂SO₄ (pH 1.7)	24	
8	2 " "	Adjusted to pH 2	24	32
9	2 " "	" " " 4	23	
10	2 " "	" " " 7	20	23
11	2 " "	" " " 9	13	
12	2 " "	Clarase‖	36	39

After extraction of the yeast by the stated method, the extracts were centrifuged, then passed through sintered glass filters, and the *clear* filtrates were further treated as described under "Hydrolytic procedure."

* 60 minutes at 15 pounds pressure.

† In terms of pyridoxine hydrochloride.

‡ The extract was steamed with the medium for sterilization, according to Atkin *et al.*[2]

§ The extract was filtered through a glass bacterial filter, then added aseptically to the steamed medium.

‖ According to Atkin *et al.*,[2] incubation being for 65 hours at 45°.

[1] Melnick, D., Hochberg, M., Himes, H. W., and Oser, B. L., *J. Biol. Chem.*, **160**, 1 (1945).

to the three known members of the B_6 group (pyridoxine, pyridoxamine, and pyridoxal), a fourth component, which is acid-labile, occurs in some natural materials such as yeast. This conclusion was based largely on the observation that strong acids, such as 2 N H_2SO_4, extract only half of the total B_6 activity from yeast, as judged by the response of *Saccharomyces carlsbergensis* and differential microbiological assay.

The table shows (Experiment 5) that, in agreement with Melnick *et al.*,[1] extracts made with 2 N H_2SO_4 apparently contain about half of the total B_6 content of the yeast (39 γ per gm.). If, however, the extract made with 2 N H_2SO_4 is further "hydrolyzed" by autoclaving in 0.055 N H_2SO_4[2] or, in general, at a pH of about 2 to 4, or treated with clarase, then the B_6 found increases considerably (Experiments 7, 8, 9, 12). These findings therefore demonstrate that the missing B_6 moiety is not destroyed by extraction with 2 N H_2SO_4 but, rather, that it is not available to *Saccharomyces carlsbergensis*.

Recent work[3] on the behavior of pyridoxal phosphate as the prosthetic group of transaminases suggests that these analytical vagaries are related to the extent of dissociation of pyridoxal phosphate from protein.

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　[2] Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **15**, 141 (1943).
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STREPTOMYCIN: THE CHEMICAL NATURE OF STREPTIDINE

Sirs:

In the course of a study of the products formed from streptomycin by acid hydrolysis, we have isolated a crystalline base $C_8H_{18}O_4N_6$ which is very probably identical with a basic degradation product of the same composition recently reported by Brink, Kuehl, and Folkers.[1] We have tentatively adopted for this substance the name, streptidine, used by these investigators.

Streptidine sulfate is most conveniently obtained by treatment of streptomycin sulfate with 4 parts of 1 N sulfuric acid for 48 hours at 45°. The resulting crystalline precipitate can be readily purified by recrystallization from water. The sulfate as well as the other crystalline salts described below has no definite melting point, but decomposes with charring above 250°. They contain water of crystallization which can be completely removed only by drying *in vacuo* at 150°. *Sulfate* (prisms), $C_8H_{18}O_4N_6 \cdot H_2SO_4$, calculated, C 26.67, H 5.60, N 23.32, S 8.88; found, C 26.63, H 5.65, N 23.0, S 8.72. *Dihydrochloride* (amorphous), $C_8H_{18}O_4N_6 \cdot 2HCl$, calculated, C 28.66, H 6.01, N 25.06, Cl 21.16; found, C 28.68, H 5.90, N 24.8, Cl 21.1. *Dipicrate* (needles or stout prisms), $C_8H_{18}O_4N_6 \cdot 2C_6H_3O_7N_3 \cdot H_2O$, calculated, C 32.53, H 3.52, N 22.75; found, C 32.46, H 3.60, N 22.6. *Carbonate* (characteristic tetrahedra), $C_8H_{18}O_4N_6 \cdot H_2CO_3$, calculated, N 25.90; found, N 25.8. The free base, obtained from the salts with excess sodium hydroxide, crystallizes in needles, but is not suitable for analytical characterization.

Streptidine is optically inactive and shows no selective absorption in the ultraviolet range. It is devoid of carbonyl oxygen, as shown by the absence, after treatment with thiosemicarbazide, of the characteristic thiosemicarbazone band at 270 mμ.[2]

Streptidine is the moiety responsible for the Sakaguchi reaction given by streptomycin.[3] The presence in streptidine of two guanidino groups has been conclusively established by the following findings. Electrometric titration of its dihydrochloride showed it to be a very strong base. Treatment of the sulfate with 5 per cent barium hydroxide solution at 95° yielded 2 moles of ammonia and the corresponding diurea $C_6H_{10}O_4 \cdot (NH \cdot CO \cdot NH_2)_2$ (decomposition point 286–287° corrected). $C_8H_{16}O_6N_4$, calculated, C 36.37, H 6.11, N 21.20; found, C 36.29, H 6.04, N 21.0.

[1] Brink, N. G., Kuehl, F. A., Jr., and Folkers, K., *Science*, **102**, 506 (1945).

[2] Evans, L. K., and Gillam, A. E., *J. Chem. Soc.*, **565** (1943).

[3] Carter, H. E., Clark, R. K., Jr., Dickman, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, **160**, 337 (1945).

More rigorous treatment with 40 per cent barium hydroxide resulted in the formation of 4 moles of ammonia and of the diamine $C_6H_{10}O_4(NH_2)_2$, which was characterized as the crystalline dihydrochloride and dipicrate. *Dihydrochloride*, $C_6H_{14}O_4N_2 \cdot 2HCl$, calculated, C 28.70, H 6.42, N 11.15, Cl 28.24; found, C 28.63, H 6.36, N 11.4, Cl 28.6, amino N 11.7. *Dipicrate*, $C_6H_{14}O_4N_2 \cdot 2C_6H_3O_7N_3$, calculated, C 33.98, H 3.17, N 17.60; found, C 33.75, H 2.97, N 17.8. In the oxidation of the diamine with periodate in alkaline medium 6 oxygen atoms were consumed.

Among the structural formulae consistent with the above results a tetra-hydroxydiguanidinocyclohexane structure, suggestive of a biogenetic relationship to inositol, would appear most satisfactory.

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THE SEPARATION OF ADENOSINETRIPHOSPHATASE FROM MYOSIN AND ITS ACTIVATION BY CREATINE

Sirs:

It has been found that by fractionating myosin under certain conditions a water-soluble enzyme may be separated from it which splits ATP to ADP and inorganic phosphate. The enzyme does not act on ADP.

All operations were carried out in a cold room at 5°. Three times precipitated myosin, prepared from rabbit muscle, was dissolved in Weber's solution[1] and dialyzed for 3 hours against 0.5 saturated $(NH_4)_2SO_4$ adjusted to pH 5.2. The precipitate was centrifuged off and stirred up in a solution containing 0.03 M ATP, pH 7.4, followed by the addition of 4 volumes of

TABLE I

Effect of Creatine and Calcium on Adenosinetriphosphatase Separated from Myosin

All samples contained 1 cc. of 0.1 M glycine, pH 9.0, 523 γ of labile P as ATP, plus 25 γ of enzyme. Total volume 1.5 cc.; incubated for 5 minutes at 38°; analyzed for inorganic phosphate (P_0) and labile P after 7 minutes hydrolysis in 1 N HCl (P_7).

Sample No.	Additions	P_0	P_7
		γ	γ
	1 mg. creatine (before incubation)	20	523
	None	50	483
	0.001 N $CaCl_2$	48	480
	1 mg. creatine	178	352
	1 " " + 0.001 M $CaCl_2$	174	354

acetone. After centrifugation, the supernatant fluid was discarded and the gummy precipitate ground in a mortar with a 0.1 M KCl solution. The mixture was centrifuged and the supernatant fluid dialyzed against water for 10 hours. The dialyzed and centrifuged solution contained an adenosinetriphosphatase which was 30 times more active per mg. of protein than myosin. The solution contained about 3 per cent of the original protein of myosin. Tiselius pictures showed one main component comprising about 85 per cent of the total protein.

The activity of the enzyme was enhanced by creatine, the half maximal effect being obtained with 1.5×10^{-3} M. There was no formation of phosphocreatine. Calcium had no activating effect (Table I). The enzyme could be readsorbed on a myosin which had been prepared at pH 6.0[2] and

[1] Weber, H. H., *Biochem. Z.*, **266,** 137 (1933).
[2] Singher, H. O., and Meister, A., *J. Biol. Chem.*, **159,** 491 (1945).

was free of adenosinetriphosphatase activity. After readsorption on myosin, calcium had an activating effect (Table II).

Water extracts prepared from rat or rabbit muscle contained an adenosinetriphosphatase which had the same properties as that separated from myosin. On dialysis this enzyme lost 80 per cent of its activity, which could be restored by creatine. This activation by creatine was not due to

TABLE II

Adsorption of Water-Soluble Adenosinetriphosphatase by Myosin

50 γ of adenosinetriphosphatase mixed with 7 mg. of myosin in 2 cc. of 4.5 per cent KCl, pH 8.0; mixture dialyzed against water for 24 hours and centrifuged; precipitate (designated as A) washed with 10 volumes of water, centrifuged, and dissolved in Weber's solution. All samples contained 1 cc. of 0.1 M glycine, pH 9.0, + 1 mg. of creatine + 498 γ of labile P as ATP in a volume of 2.0 cc. Incubated and analyzed in the manner as described in Table I.

Sample No.	Additions	P_0	P_7
		γ	γ
1	Before incubation	25	498
2	2 mg. myosin before adsorption + 0.001 M CaCl$_2$	27	495
3	2 mg. A	85	438
4	2 " " + 0.001 M CaCl$_2$	120	402

formation of phosphocreatine. Calcium had no activating effect. This enzyme could also be adsorbed on myosin and again separated from myosin by methods described in this paper. These findings would indicate that adenosinetriphosphatase is present in muscle partly in the free form and partly adsorbed on myosin.

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TABLE III
Growth Results on Various Diets

Group No.	Ration No. (Table II)	Diet	Added niacin*	Added tryptophane†	Gain per wk. for 4 wks. (range)
					gm.
1	1	Sucrose, casein			15 (12–16)
2	2	Dextrin, "			23 (20–28)
3	3	Glucose, "			21 (19–23)
4	4	Corn-starch, casein			26 (24–29)
5	5	Sucrose, casein, gelatin			3 (2–5)
6	5	" " "	+		18 (16–21)
7	5	" " "		+	24 (21–28)
8	6	Dextrin, " "			10 (8–14)
9	6	" " "	+		24 (21–29)
10	6	" " "		+	21 (20–30)
11	7	Glucose, " "			11 (7–14)
12	7	" " "	+		21 (19–23)
13	7	" " "		+	20 (19–20)
14	8	Corn-starch, casein, gelatin			10 (8–11)
15	8	" " "	+		20 (18–22)
16	8	" " "		+	22 (20–24)
17	9	Sucrose, wheat gluten, lysine			17 (15–18)
18	9	" " " "	+		16 (15–17)
19	10	" " " fibrin			4 (3–5)
20	10	" " " " lysine			14 (13–15)
21	10	" " " " "	+		20 (17–24)
22	10	" " " " "		+	21 (19–23)
23	11	" " " " gelatin, lysine			6 (5–7)
24	1	" "	+		18 (16–20)
25	11	" "		+	23 (20–25)
26	12	" "			5 (4–5)
27	12	" "	+		20 (14–24)
28	12	" "		+	14 (12–16)
29	1	Sucrose, casein, lysine, histidine			20 (16–24)
30	13	" " fibrin			2 (2–3)
31	13	" " " lysine, histidine			4 (3–6)
32	13	" " " " "	+		20 (13–26)
33	13	" " " " "		+	22 (20–24)
34	14	" " " gelatin, lysine, histidine			3 (2–5)
35	14	" "	+		18 (16–19)
36	14	" "		+	26 (22–29)
37	15	" "			5 (3–5)
38	15	" "	+		15 (14–17)
39	15	" "		+	22 (21–23)
40	15	" "		+	24 (22–27)
41	16	Sucrose, casein, zein, lysine, histidine			3 (2–5)
42	16	" " " " "	+		22 (21–24)
43	16	" " " " "		+	23 (20–25)
44	17	Dextrin, " " " "			12 (8–16)

TABLE II

Composition of Basal Rations

Ration constituent	Ration No.																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Sucrose	84				78								82	79	76	81	
Dextrin		84				78			76	74	68	64					81
Glucose			84				78										
Corn-starch				84				78									
Casein*	9	9	9	9	9	9	9	9					9	9	9	9	9
Wheat gluten†									17	17	17	17					
Fibrin hydrolysate										2	2	2	2	2	2		
Gelatin					6	6	6	6			6	10		3	6		
Zein	3	3	3	3	3	3	3	3					3	3	3	3	3
Corn oil	4	4	4	4	4	4	4	4	3	3	3	3	4	4	4	3	3
Salts IV‡	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
l(−)-Cystine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2				0.2	0.2	0.2	0.2	0.2	0.2

Vitamins were added to all rations at adequate levels; see the text.

* Casein three times extracted with 95 per cent ethanol.
† 17 per cent wheat gluten provided 15 per cent protein.
‡ Phillips, P. H., and Hart, E. B., J. Biol. Chem., **109**, 657 (1935).

TABLE III—*Concluded*

Lysine and histidine refer to $l(+)$-lysine and $l(-)$-histidine respectively, which were added as the hydrochlorides in such amounts as to supply the minimum requirement of each; *i.e.*, 1.0 and 0.4 per cent respectively. Fibrin refers to fibrin hydrolysate.

* Niacin added at a level of 1.5 mg. per cent.

† Tryptophane added at a level of 50 mg. per cent except for Group 40 for which the level was 100 mg. per cent.

this growth depression as adequately as before and since larger amounts of tryptophane seemed to give somewhat better growth (see Table III, Groups 34 through 40).

Although the 17 per cent wheat gluten rations contain a little more tryptophane than the 9 per cent casein ration, *i.e.* 120 and 108 mg. per cent respectively, results obtained with these two proteins plus tryptophane-free proteins were remarkably comparable.

From the above results it is quite clear that rations which are deficient in nicotinic acid and marginal in their tryptophane content are inadequate for good rat growth.

Since this is the condition that prevails in corn-supplemented rations, the effect of zein, the principal protein in corn, was tested by adding it at a level of 3 per cent to a 9 per cent casein ration at the expense of carbohydrate. (This level of zein is about that contained in the corn-supplemented ration.) The very poor growth obtained with zein (Table III, Group 41) unless nicotinic acid or tryptophane is added (Table III, Groups 42 and 43) extends the observations made with gelatin and acid-hydrolyzed fibrin and indicates quite strongly that the deleterious action of corn in creating a nicotinic acid deficiency or increased tryptophane requirement in the rat is intimately related to the protein or more correctly to the distribution of the α-amino acids in the protein of this cereal.

DISCUSSION

The addition of corn grits to diets containing fibrin and egg albumin reduced the protein level from 15 to 12.6 per cent and caused a very slight decrease in the growth of rats. This decrease, however, was not prevented by nicotinic acid, and cannot be compared to the drastic growth retardation which prevails when casein is used at comparable levels. The protective action of fibrin and 15 per cent egg albumin can be attributed to their respective tryptophane contents. Growth on the 10 per cent egg albumin ration was quite poor and consequently little can be said concerning the effect of corn grits.

The present observations confirm the findings of Hegsted *et al.* (7) that the tryptophane requirement of the growing rat under normal conditions

is considerably less than the level of 0.2 per cent set by Rose (8). It should be noted, however, that the diet used by Rose in establishing this level was a relatively high fat, high calorie diet, while the diets employed here are low in fat. That the definition of normal conditions can be somewhat ambiguous is exemplified by the above results in which the tryptophane requirement of the rat on a nicotinic acid-free diet was increased from about 0.1 per cent or less of the ration to about 0.15 per cent simply by increasing the concentration of total protein. The use of dextrin in place of sucrose apparently decreases the tryptophane requirement. Since the rat does not require a dietary source of nicotinic acid, the use of the ration free of this vitamin could not be considered abnormal.

Although the growth-inhibiting effect of corn can be attributed to the character of the protein which it contains, the solution of the problem of why an increased concentration of certain amino acids on a nicotinic acid-low diet gives poor growth and precipitates an increased need for tryptophane remains to be answered.

Of considerable interest is the finding by Martin (9) that 2 per cent succinylsulfathiazole in diets containing casein, enzymatic casein digest, or a mixture of ten essential amino acids respectively resulted in great weight loss and death when the ten essential amino acids were the source of nitrogen. Growth on the other two diets was not markedly affected. Martin interprets these results by suggesting that intestinal bacteria must synthesize amino acids essential to the host which are not present in the mixture of the ten known essential amino acids.

If it is true that intestinal bacteria play a rôle in the synthesis of tryptophane, then it is reasonable to think that the intestinal flora could be so altered in its synthetic capacity as to be reflected in an increased dietary requirement. The presence or absence of nicotinic acid could be influential in such an alteration. It is possible too that the bacterial flora under certain conditions destroys or prevents the utilization of tryptophane, thereby increasing the dietary requirement from about 0.1 per cent to about 0.15 per cent.

Hier *et al.* (10) reported a failure to realize optimum growth with gelatin supplemented with essential amino acids in which gelatin is lacking. This growth failure was attributed to the toxicity of certain specific amino acids. Gelatin as a substitute for plasma proteins has been shown to have a limited ability to contribute to the synthesis of plasma protein and hemoglobin in dogs and toxicities are evident on prolonged administration (11).

A direct toxicity would hardly explain the present results, since good growth can be obtained when relatively small amounts of tryptophane and nicotinic acid are added to the ration.

The present findings suggest that the effectiveness of synthetic mixtures

of amino acids or protein digests might be impaired if precautions are not taken to supply adequate amounts of tryptophane and nicotinic acid. It is also suggested that the usually effective supplemental action of certain proteins may be thwarted under conditions of nicotinic acid deficiency.

SUMMARY

The deleterious action of corn grits in a synthetic diet was prevented when fibrin, egg albumin, or soy bean globulin was used in place of casein. This protection is attributed directly to the more adequate amount of tryptophane supplied by these proteins.

A syndrome analogous to that produced by corn was duplicated with non-corn-containing rations by adding tryptophane-free proteins or an acid-hydrolyzed protein to nicotinic acid-low rations which also contained marginal amounts of tryptophane. In a similar manner the growth-inhibiting effect of corn was shown to be related to the nature of its protein.

Possible explanations of these observations are discussed.

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THE ISOLATION AND IDENTIFICATION OF A NATURAL PRECURSOR OF CHOLINE

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Previous papers (1, 2) have described two artificially produced choline-requiring mutants of *Neurospora crassa*. These mutants are designated as strain 34486, or *cholineless-1*, and strain 47904, or *cholineless-2*. Each strain carries a mutation of a different single gene concerned with the synthesis of choline. The effect of the mutations is a complete (strain 34486) or partial (strain 47904) failure in the ability of the mutants to synthesize choline. No, or little, growth takes place on an unsupplemented basal medium, but both strains attain a growth rate comparable to that of the wild type in choline-supplemented media. In addition to choline, a number of choline analogues are active in promoting growth of the mutants (2).

In the preceding paper (2) the accumulation in cultures of *cholineless-2* of a substance which promotes the growth of *cholineless-1* was reported. Growth curves obtained with choline-free concentrates of the substance resembled those given by monomethylaminoethanol. Because of the failure to obtain an active picrolonate or chloroplatinate from the concentrates, however, it was tentatively concluded that the substance is not identical with monomethylaminoethanol. The isolation and identification of the factor have now been completed, and the previous conclusion has been found to be erroneous. The substance is, in fact, N-monomethylamino-ethanol, $CH_3NHCH_2CH_2OH$. The earlier failure to obtain active precipitates with picrolonic and chloroplatinic acids was in all probability due to interfering impurities in the original concentrates.

The accumulation of monomethylaminoethanol by strain 47904 is evidently the consequence of a block which prevents its normal conversion to choline and indicates that this substance is an intermediate in the synthesis of choline by *Neurospora*. The fact that monomethylaminoethanol can replace choline for strain 34486 but not for strain 47904 is consistent with this interpretation and proves that the synthesis is blocked at different stages in the two mutants. The results thus support the hypothesis that a 1:1 correspondence exists between genes and biochemical reactions (3).

EXPERIMENTAL

Methods—The basal medium used in these experiments was that previously described (1). Unless otherwise indicated, growth studies were

carried out in 125 ml. Erlenmeyer flasks containing 20 ml. of medium. The incubation period was 72 hours at 25°, following which the mycelium was dried and weighed. For further details see the paper of Horowitz and Beadle (1).

Accumulation of Precursor—The production of the active substance by strain 47904 can be demonstrated by adding a few ml. of medium on which this strain has grown to a larger quantity of the basal (choline-free) medium, sterilizing, and inoculating with strain 34486. The growth which results indicates the presence of the choline precursor. In this test, advantage is taken of the fact that strain 34486 is one-fifth to one-tenth as sensitive
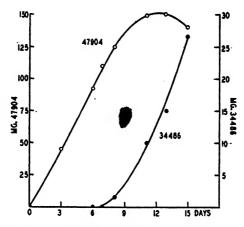
FIG. 1. Accumulation of choline precursor in cultures of strain 47904. Open circles, growth of strain 47904 with time; solid circles, increase of precursor with time, as measured by 72 hour growth of strain 34486. For further description see the text.

to choline as is strain 47904. By proper adjustment of the concentration of choline in the medium used for growing strain 47904, the amount of choline subsequently carried over in the test sample can be made negligible in terms of growth activity for strain 34486.

Filtrates of the medium in which strain 34486 has grown show no activity for strain 47904 beyond what may be expected from the choline carried over.

The increase of the active material with time is shown in Fig. 1. In this experiment, strain 47904 was inoculated into 250 ml. Erlenmeyer flasks containing 25 ml. of basal medium plus 1 γ of choline. The flasks were incubated at 25°, and the mold was harvested at the indicated time intervals, dried, and weighed. The filtrates were tested for active material. 5 ml. of filtrate were added to 20 ml. of the basal medium, autoclaved, and inoculated with strain 34486. After 72 hours incubation at 25°, the mold

was harvested, dried, and weighed. Fig. 1 shows the growth of strain 47904 with time, together with the increase in activity of the filtrate as measured by the growth of strain 34486. It will be noted that no activity was observed in the filtrate before 7 days. This apparent lag reflects the limit of sensitivity of the test method as well as the fact, established in later experiments, that the active material is more highly concentrated in the mold than in the medium. The findings suggest that an intracellular threshold may exist for the substance, below which no appreciable spilling

TABLE I

Procedure for Isolation of Monomethylaminoethanol from Neurospora

Treatment	Active fraction
Mycelium homogenized with water in Waring blendor, extracted with boiling water; filtered	Filtrate
Filtrate concentrated under reduced pressure, poured into 5 volumes methanol; chilled in refrigerator; ppt. filtered off	"
Filtrate concentrated to about 100 ml., 1 volume saturated aqueous Reinecke salt added; chilled in refrigerator; filtered	"
Excess reineckate ion pptd. from filtrate with silver nitrate; filtered	"
Filtrate brought to pH 10 with NaOH, distilled under reduced pressure until ammonia-free (Nessler's test); made 2 N in NaOH, extracted with 1 volume H_2O-saturated butyl alcohol on mechanical shaker; butyl alcohol extraction repeated 7 times	Butyl alcohol phase
Butyl alcohol extract distilled under reduced pressure (38 mm. Hg)	Distillate
Distillate extracted with dilute HCl	Aqueous phase
Aqueous phase concentrated to few ml., made strongly alkaline with KOH, distilled under reduced pressure	Distillate
Alkaline distillate brought to pH 6.5 with saturated alcoholic picrolonic acid; solvents distilled off; residue recrystallized 3 times from absolute alcohol	Yellow needles

over into the medium occurs. The decrease in dry weight of strain 47904 after 13 days incubation is probably due to autolysis following exhaustion of the medium, a phenomenon which is regularly observed in old cultures of *Neurospora*.

Isolation of Monomethylaminoethanol—For the isolation of the active factor, strain 47904 was grown in 5 gallon Pyrex carboys containing 16 liters of basal medium plus 2 mg. of choline chloride. The carboys were incubated at 25° under forced aeration (10 liters of air per carboy per minute). At the end of 10 days the mold was filtered off through cheese-cloth

and excess medium squeezed out.   400 to 500 gm. of moist mycelium were usually obtained from each carboy.   With the growth of strain 34486 as an indicator of the active substance, the mycelium was fractionated by the procedure shown in Table I.   The substance can be isolated from the medium by essentially the same procedure, but in this case the yield per carboy is less than 25 per cent that obtained when the mycelium is used as the starting material.

The active factor was isolated in the form of its picrolonate, crystallizing as yellow needles from alcohol; m.p. 225–226° (uncorrected) with decomposition.   Yield, 300 mg. per kilo of moist mold.   Elementary analysis showed the following.

$$C_3H_9NO \cdot C_{10}H_8N_4O_5.$$ Calculated.   C 46.00, H 5.05, N 20.65
            Found.   " 45.94, " 5.28, " 20.45

The melting point of synthetic monomethylaminoethanol picrolonate was found to be identical with that of the isolated material.   The mixed melting point showed no depression.   The observed melting point is considerably below the value of 239° reported by Matthes (4), but repeated recrystallization of both the synthetic and natural substances has failed to raise their melting points above 226°.

The biological activities of the natural and synthetic compounds were found to be the same.   Both are inactive for strain 47904, which produces the substance, and are equally active, within the limits of experimental error, for strain 34486 (Fig. 2).   The data of Fig. 2 were obtained with the undecomposed picrolonates.   Separate experiments have shown that the picrolonate ion has no effect on the growth of the mutants in the concentrations used here.

The finding that monomethylaminoethanol recrystallized through the picrolonate is inactive for strain 47904 corrects a previous report (2) that monomethylaminoethanol has activity for this mutant.   The earlier finding apparently resulted from the presence of a small amount of active impurity in the sample of the free base used in the experiments.   This sample, b.p. 158–159°, was prepared from commercial reagents by the method of Knorr and Matthes (5).

The presence of an impurity in the sample was first suspected following the isolation from strain 47904 of the natural substance in the form of its mercuric chloride double salt.   This salt was the first crystalline derivative of the natural precursor to be obtained.   On the basis of melting point (126°), elementary analysis, and x-ray photographs (kindly taken by Professor Linus Pauling and Mr. A. M. Soldat) it was determined that the material was monomethylaminoethanol-mercuric chloride double salt. However, the biological activity of the natural material for strain 34486 was

COMPARATIVE TOLERANCE TO MIXTURES OF NATURAL AND RACEMIC AMINO ACIDS ON INTRAVENOUS INFUSION IN THE DOG

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Madden and his collaborators (1–3) have reported that certain mixtures of crystalline amino acids are well tolerated when infused intravenously at high rates into dogs, whereas protein hydrolysates must be given fairly slowly to be tolerated. They have also found that glycine added to mixtures of pure amino acids improves their tolerance. They further report that glutamic acid is particularly likely to produce vomiting and suggest that the lower tolerance to the hydrolysates may be due, in part at least, to their content of this amino acid. Unna and Howe (4) have corroborated this observation concerning glutamic acid and have also demonstrated that aspartic acid has an equal emetic effect. Madden *et al.* (5) more recently have also reported that *dl*-aspartic acid in mixtures of crystalline amino acid induces vomiting.

In this communication we wish to record striking differences in the tolerance of mixtures of racemic and natural amino acids, to elaborate on some of Madden's findings, and to present a preliminary study of the tolerance to single amino acids.

EXPERIMENTAL

Healthy adult mongrel dogs maintained on a diet of Purina dog chow were given single intravenous infusions of solutions of the mixtures listed in Table I, of variations of these mixtures, and of single amino acids. Food was withdrawn from the cages in the afternoon of the day preceding the experiment. The rates of infusion were carefully controlled by use of a variable speed infusion pump designed by Mr. Michael Kniazuk of the Merck Institute. With this instrument an 8 per cent solution of amino acids may be given to a 10 kilo dog at a maximum rate of 35 mg. of nitrogen per kilo per minute. Pulse and temperature were recorded at intervals during the infusion and for 2 hours thereafter. No dog was used more often than twice weekly.

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Vomiting was used as the criterion of non-tolerance. The vomiting was preceded by a short period of nausea, retching, and a slight fall in rectal temperature. On continuation of the infusion the vomiting often reoccurred in short intervals. However, after the termination of the infusion, vomiting ceased and the animals recovered quickly within 2 hours. No elevation in temperature was observed during the infusion. Only occasionally a rise of 1° or 2° was encountered 1 to 2 hours after the infusion. Such temperature reactions were presumably due to contamination with pyrogens, and not to the amino acids *per se*.

TABLE I

Per Cent Composition of Mixtures of Amino Acids

	Mixture I	Mixture II	Mixture III	Mixture IV	Vuj mixture	Vuj-N mixture
l(+)-Arginine HCl............				10.8	8.0	8.0
l(+)-Histidine HCl·H₂O.....				5.3	4.0	4.0
l(+)-Isoleucine..............	14.2			9.7		7.8
dl-Isoleucine................		16.6	16.5		10.8	
l(+)-Leucine	32.0		23.5	21.7	15.4	17.5
dl-Leucine...................		37.4				
l(+)-Lysine HCl.............				16.1	12.3	12.3
l(−)-Methionine.............	9.2			6.2		5.5
dl-Methionine		10.7	9.2		6.1	1.2
l(−)-Phenylalanine..........	16.0			10.8		8.7
dl-Phenylalanine.............		18.7	10.6		6.9	
l(−)-Threonine..............	3.2			2.2		1.7
dl-Threonine................		3.7	16.5		10.8	7.4
dl-Tryptophane..............			2.7		1.8	1.8
l(+)-Valine.................	11.1			7.6		6.1
dl-Valine		12.9	21.0		13.9	
Glycine.....................					10.0	10.0
Total......................	85.7	100.0	100.0	90.4	100.0	92.0

The amino acids were dissolved in distilled water by heating on a steam bath to 70–80°. The solutions in all cases with the exception of *dl*-phenylalanine (to be discussed later) were adjusted to pH 6 to 7 with strong sodium hydroxide solution. The nitrogen content of the complete amino acid mixtures (those containing all of the essential amino acids) and of the hydrolysates tested varied from 1.0 to 1.1 gm. per 100 cc. The total amount of nitrogen administered in these preparations was usually approximately 220 mg. per kilo of body weight. Correspondingly smaller amounts of the incomplete mixtures were given.

In Table I the amino acid composition of some of the infusion mixtures

is shown. Mixture I is the monoaminomonocarboxylic fraction of a protein hydrolysate obtained from a casein hydrolysate by Dakin's butanol extraction procedure. Its amino acid composition was determined by microbiological assay. The unidentified portion of this mixture containing 15 per cent of the total nitrogen is possibly to a large extent natural nonessential amino acids. Mixture II is a mixture of the essential racemic acids in the same proportion in which the natural acids are found in the butanol extract, whereas Mixture III is a mixture of the monoaminomonocarboxylic acids in the proportions in which they are found in Madden's Vuj mixture (6). No. IV is a mixture of natural amino acids made by adding the pure basic amino acids to Mixture I. The fifth mixture is Madden's Vuj mixture which has, to some extent, been used clinically. The final mixture is obtained by addition of certain racemic amino acids and glycine to Mixture IV to make it resemble the Vuj mixture with respect to natural amino acid content. Because of this close resemblance and because it consists predominantly of natural amino acids, it has been given the designation Vuj-N mixture.

RESULTS AND DISCUSSION

The monoaminomonocarboxylic fraction (Mixture I) was infused at rates varying from 5 to 22 mg. of N per kilo per minute in a series of seventeen experiments with eleven dogs without causing vomiting or any apparent nausea. However, when a mixture of the same essential amino acids in the same proportions but in the racemic form (Mixture II) was given to three dogs at rates between 1 and 2 mg. of N per kilo per minute, all of these animals vomited repeatedly. In a like manner, Mixture III containing racemic monoaminomonocarboxylic acids and $l(+)$-leucine in the proportions found in the Vuj mixture produced violent retching for twelve of fourteen infusions when it was administered at rates of 2 to 4 mg. of N per kilo per minute. Again, when the $l(+)$-leucine of Mixture III was replaced by dl-leucine (Mixture IIIa) two of four dogs vomited at the rate of 2 mg. of N per kilo per minute.

The mixture of nine natural essential amino acids (Mixture IV) was used in nine infusions at rates of 20 to 35 mg. of N per kilo per minute. Only two of the animals vomited and in these experiments the rates of infusion were 30 and 31 mg. of N per kilo per minute. Similarly, when this mixture was fortified with about 10 per cent racemic amino acids and 10 per cent glycine (Vuj-N mixture) it was extremely well tolerated, producing no vomiting for eighteen infusions at rates of 6 to 30 mg. of N per kilo per minute. In contrast, the Vuj mixture produced vomiting in eight of a series of sixteen infusions at rates between 5 and 6.5 mg. of N per kilo per minute. Table II summarizes the results of the experiments

which show the striking differences in the tolerance to natural and racemic amino acids.

Although these great differences in tolerance may not be considered evidence of non-utilization of the d forms of the amino acids, they become of prime importance in parenteral amino acid alimentation, when speed of infusion may be advantageous.

The effect of glycine in increasing the tolerance of the dog to mixtures consisting predominantly or entirely of racemic amino acids is not great. Three dogs vomited when they received Mixture III plus 10 per cent glycine (Mixture VII) at rates of 2.5 to 3.5 mg. of N per kilo per minute.

TABLE II

Tolerance to Mixtures of Natural and Racemic Amino Acids

No. of dogs	Mixture No.	Description of mixture	Nitrogen, total	Nitrogen rate	No. of infusions	No. of vomitings
			mg. per kg.	*mg. per kg. per min.*		
11	I	Natural monoaminomonocarboxylic acids	110–120	5–22	17	0
3	II	Racemic amino acids corresponding to Mixture I	120	1– 2	3	3
9	III	Monoaminomonocarboxylic acids as found in Vuj	120	2– 4	14	12
4	IIIa	Mixture III with *dl*-leucine replacing *l*(+)-leucine	120	2.0	4	2
7	IV	Natural amino acids	216	20–35	9	2
8	Vuj	Mixture containing 50% racemic amino acids	220	5– 6.5	16	8
11	Vuj-N	Natural amino acids + 10% racemic amino acids + 10% glycine	220	6–30	18	0

Addition of 10 per cent glycine to Mixture IIIa (Mixture VIII) again showed little or no increase in tolerance, since three of four dogs vomited at rates of 2.5 to 3.5 mg. of N per kilo per minute. When 25 per cent of glycine was added (Mixture IX) five of six dogs vomited at rates of 3 to 4 mg. of N per kilo per minute.

That glycine does, however, greatly increase the tolerance for racemic amino acids in some mixtures of racemic and natural acids is demonstrated by a series of experiments in which the Vuj-N mixture minus glycine (Mixture X) was used. As has been previously recorded, a mixture of natural amino acids is tolerated at rates up to 30 mg. of N per kilo per minute. Similarly, when 10 per cent racemic acids and 10 per cent glycine are added to this mixture it may again be given at a rate of 30 mg. of N per kilo per minute without nauseating effects. However, when the

glycine is omitted vomiting occurs quite frequently at rates as low as 9 mg. of N per kilo per minute. Forty infusions were carried out at rates between 6 and 30 mg. of N per kilo per minute. In twenty-one of the forty vomiting occurred. Closer examination of the data indicates that this mixture behaves somewhat anomalously, *i.e.*, the incidence of vomiting was greater upon infusion at rates of 9 to 11 mg. when seven of ten dogs vomited than at 18 to 22 mg., when vomiting occurred in only seven of fourteen. As an example of individual performance Dog 571 vomited when the infusion rate was 11 mg. of N per kilo per minute but in two infusions at 21.5 mg. vomited only once. This great individual variation was not encountered with the other mixtures used.

TABLE III

Effect of Glycine on Tolerance to Amino Acid Mixtures

No. of dogs	Mixture No.	Description of mixture	Nitrogen, total	Nitrogen rate	No. of in-fusions	No. of vomit-ings
			mg. per kg.	*mg. per kg. per min.*		
9	III	Monoaminomonocarboxylic acids as found in Vuj mixture	120	2 – 4	14	12
3	VII	Mixture III + 10% glycine	120	2.5– 3.5*	3	3
4	IIIa	" " with *dl*-leucine replacing *l*(+)-leucine	120	2	4	2
4	VIII	Mixture IIIa + 10% glycine	120	2.5– 3.5*	4	3
6	IX	" " + 25% "	120	3 – 4*	6	5
7	IV	Natural amino acids	216	20 –35	9	2
11	VI	Mixture IV + 10% racemic amino acids + 10% glycine	220	6 –30	18	0
12	X	Vuj-N mixture minus glycine	214	6 –30	40	21

* These figures do not include the glycine nitrogen.

The results of the experiments showing the effect of glycine upon the tolerance to amino acid mixtures are summarized in Table III.

The poor tolerance to the racemic amino acid mixtures (Mixtures II and III) might be a cumulative effect of all or, on the other hand, might be due to a comparatively great emetic effect of one or a limited number of them. Accordingly, each of these racemic acids was infused singly at several times the rates and in at least twice the total amounts that they were given in the mixtures. The results, found in Table IV, show that each amino acid is remarkably well tolerated. Even *dl*-methionine, by far the worst offender, could be injected at 4 times the rate and in twice the total quantity that it is given in the Vuj solution when infused at the maximum tolerated speed of 6 mg. of N per kilo per minute.

Owing to its low solubility, dl-phenylalanine was given in solution at pH 8.5. This amino acid produced vomiting in one of three experiments when a total of 1 gm. per kilo of body weight was administered at 2.8 mg. of N per kilo per minute. This positive result is probably without significance, however, since glycine which is normally innocuous produced vomiting in one of two experiments at pH 8.5.

TABLE IV

Tolerance to Single Amino Acid

No. of dogs	Description of material	Amino acid		Nitrogen		No. of infusions	No. of vomitings
		gm. per kg.	*mg. per kg. per min.*	*mg. per kg.*	*mg. per kg. per min.*		
2	dl-Isoleucine	1.0	33	105	3.5	2	0
2	dl-Leucine	1.0	33	105	3.5	2	1*
2	dl-Methionine	0.2	10.6	18	1.0	2	0
2	"	0.6	16–22	5.6	1.6– 2.1	2	2
3	dl-Phenylalanine	1.0	33	8.4	2.8	3	1†
4	dl-Threonine	1.0	33	116	3.9	4	2
2	dl-Tryptophane	0.06	3.2	8	0.4	2	0
2	dl-Valine	1.0	33	119	4.0	2	0
9	Mixture III, monoamino-monocarboxylic acid as found in Vuj			120	2 – 4	14	12
5	Mixture III minus dl-methionine			70–100	2.5– 5	5	2
2	Mixture III minus dl-threonine			98	2 – 3	2	2
4	Mixture III minus dl-methionine and dl-threonine			102	4 – 5	4	1
2	" "			102	7	2	2
11	Mixture I, natural mono-aminomonocarboxylic acids			110–120	5 –22	17	0

* Vomiting possibly due to the large volume of solution given.

† Vomiting probably due to the pH of the solution (8.5).

Since vomiting was encountered with alkaline solutions, no attempt was made to increase the solubility of dl-leucine by forming the sodium salt. Accordingly, this amino acid was infused in a 1.2 per cent solution. In this connection it is of interest to note the volume of solution which may be infused without apparent ill effects. Two dogs each received 80 cc. of solution per kilo over a period of 30 minutes. One vomited a few minutes after the infusion; in the second there was no reaction. Because of the large volume of solution infused the positive reaction in the first dog cannot be definitely ascribed as an effect of the dl-leucine.

To eliminate the possibility that the poor tolerance of the racemic amino acids might be due to either or both of the two least well tolerated of the amino acids, methionine and threonine, these acids have been omitted from the infusion mixture. The results of these experiments are shown in Table IV. When methionine is eliminated from Mixture III there is a possibility of a slight increase in tolerance to the mixture. When threonine is left out, there is apparently no appreciable change. However, when both are removed, the rate at which the mixture may be given without inducing vomiting seems to be appreciably increased although the ceiling of tolerance is still quite low in comparison with that of the natural amino acids.

SUMMARY

1. Mixtures of natural amino acids can be given to dogs intravenously at much higher rates without inducing vomiting than can similar mixtures containing racemic amino acids.

2. Glycine causes a definite increase in the tolerance of the dog to infusion of certain mixtures containing racemic amino acids.

3. Of the single racemic amino acids tested, *dl*-methionine is the most poorly tolerated. The poor tolerance of the mixtures of amino acids is, however, undoubtedly due to cumulative effect of the racemic acids present.

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THE EFFECT OF PROTEIN ON THE NICOTINIC ACID AND TRYPTOPHANE REQUIREMENT OF THE GROWING RAT*

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Previous work (1, 2) has clearly established that the presence of corn in a synthetic low protein diet alters the dietary nicotinic acid and tryptophane requirement of the growing rat. This relationship between nicotinic acid and tryptophane is not peculiar to corn-supplemented rations, since similar effects can be demonstrated (3) with non-corn rations which are low in both tryptophane and nicotinic acid.

Since tryptophane is most directly concerned with this syndrome, the present report deals with a more extensive examination of proteins other than casein which contain different amounts of tryptophane (*i.e.* egg albumin, fibrin, and soy bean globulin) and their influence in counteracting the effect of corn. Further evidence is also presented to show that the interchangeable rôle of nicotinic acid and tryptophane is intimately related to the nature of the total amino acid content of the ration.

<div align="center">EXPERIMENTAL</div>

Egg albumin and fibrin which were obtained commercially contained practically no nicotinic acid. Soy bean globulin was prepared according to the method of Teresi *et al.* (4), by extracting ground Illini type soy beans with 10 per cent NaCl solution, and dialyzing the extract in cold running water at temperatures not exceeding 15°. The globulin which precipitated after 48 to 72 hours was removed and dried after washing with ethanol and ether. The globulin was autoclaved at 15 pounds pressure for 1 hour. The protein so prepared was practically free from nicotinic acid.

Each of the proteins was analyzed for total protein (N × 6.25) and sufficient quantities were used to supply 10 and 15 per cent of total protein

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in the diet (Table I). The composition of the different basal diets was as follows:

	Low protein	High protein
Sucrose	83	78
Protein	10	15
Corn oil	3	3
Salts IV	4	4
$l(-)$-Cystine	0.15	0.15

The final ration was prepared by mixing 60 parts of each of the above rations and 40 parts of corn grits. The vitamins in these and ensuing rations were added in the following amounts per 100 gm. of ration: thiamine 0.2 mg., riboflavin 0.3 mg., pyridoxine 0.25 mg., calcium pantothenate 2.0 mg., choline 100 mg., 2-methyl-1,4-naphthoquinone 0.1 mg., inositol 10 mg., and biotin 0.01 mg. Vitamins A and D were supplied as halibut liver oil, 2 drops every week, together with vitamin E, 1 mg. per week per rat. The basal rations in this and the following series contained practically no nicotinic acid unless added as indicated.

Weanling male rats of the Sprague-Dawley strain, 35 to 45 gm. in weight, were used throughout. At least three rats were used in each group in all experiments, and most groups were repeated to confirm the results. The range of growth for all animals is given in parentheses in Tables I and III. The tryptophane content of the rations was calculated from the figures available in the literature (5) (the value for casein of 1.1 to 1.2 per cent has been verified in this laboratory).

When egg albumin, fibrin, and soy bean globulin were used as proteins, the addition of corn grits had no growth-inhibiting effect at either the lower or upper level of protein, and the addition of nicotinic acid did not cause any significant increase in growth. This is in marked contrast to the results obtained with casein at comparable levels (2). This difference is undoubtedly dependent upon the higher tryptophane content in these proteins as compared with casein. Lysine, arginine, glycine, and methionine were tried at levels of 0.5 per cent in corn-supplemented diets with casein as the protein but all were ineffective in promoting growth. This further emphasizes the specificity of tryptophane.

Since previous results (3) indicated that alterations in the intestinal flora might be responsible for the action of corn in retarding growth, further attempts were made to duplicate these observations with non-corn-containing rations. It was further hoped that by this means the rôle of nicotinic acid in the apparent intestinal synthesis of tryptophane could be clarified.

Originally it had been planned to prepare a ration analogous to the corn-supplemented ration with respect to casein (*i.e.* a 9 per cent level of

casein) but since this level of casein supplies a suboptimum amount of several of the essential amino acids other than tryptophane it was decided also to prepare a second basal ration with 6 per cent gelatin in addition to 9 per cent casein. Although experiments conducted at about the same time with wheat gluten and gelatin (3) indicated that undesirable growth might occur when nicotinic acid and tryptophane are simultaneously low, it seemed warranted to suppose that a ration composed of 9 per cent casein and 6 per cent gelatin would allow better growth than a ration which contained only 9 per cent protein as casein. It seemed that this ration had

TABLE I

Influence of Different Proteins on Growth-Retarding Effect of Corn Grits

Sucrose basal diet			Protein content (N × 6.25)	Niacin content	Trypto-phane content	Gain per wk. (range)
Protein	Corn grits	Niacin*				
			per cent	*mg. per cent*	*mg. per cent*	*gm.*
10% fibrin.................	−	−	10.0	<0.01	340	17 (12–21)
10% "	+	−	9.6	0.25	226	19 (17–23)
10% "	+	+	9.6	1.25	226	19 (18–20)
15% "	−	−	15.0	<0.01	510	27 (21–32)
15% "	+	−	12.6	0.25	338	23 (20–28)
15% "	+	+	12.6	1.25	338	23 (21–24)
10% egg albumin..........	−	−	10.0	<0.01	140	15 (13–18)
10% " "	+	−	9.6	0.25	106	13 (11–14)
10% " "	+	+	9.6	1.25	106	14 (12–15)
15% " "	−	−	15.0	<0.01	210	19 (18–19)
15% " "	+	−	12.6	0.25	148	17 (14–21)
15% " " :..........	+	+	12.6	1.25	148	18 (18–19)
15% soy bean globulin......	−	−	15.0	<0.01	240	12 (10–16)
15% " " "	+	−	12.6	0.25	166	12 (11–13)
15% " " "	+	+	12.6	1.25	166	12 (7–16)

* Niacin was added at a level of 1 mg. per 100 gm. of ration.

the further advantage of increased total protein without disturbance of the tryptophane content of the ration, which remained at 108 mg. per cent.

However, the growth results obtained with the rations supplying the two levels of protein were quite different than what had been anticipated. Growth on the 9 per cent casein diet was surprisingly good regardless of the addition of either nicotinic acid or tryptophane, whereas growth on the ration containing 6 per cent gelatin in addition to the 9 per cent casein was extremely poor. More important, however, is the fact that nicotinic acid at a level of 1.5 mg. per cent or tryptophane added at a level of 50 mg. per cent counteracted this poor growth in a manner exactly analogous to

that experienced with the corn rations. The composition of these rations and the others used in the rest of the experiments is shown in Table II. The growth results described above are summarized in Table III, Groups 1 through 16.

Inasmuch as the kind of carbohydrate employed in the corn rations had markedly influenced the extent of the growth depression on the corn rations, attempts were made to see whether the same carbohydrate effects could be observed when non-corn rations were employed. It is evident (Table III, Groups 8, 11, and 14) that dextrin, glucose, or corn-starch did not have as marked an influence in counteracting the growth depression resulting from the casein-gelatin diet as they had in the previous corn-supplemented rations (3). This might be due to the slightly lower tryptophane content of the present rations.

Previous results with a wheat gluten-gelatin ration (3) had been thought to be due at least in part to a concomitant lysine deficiency. However, analytical data indicated that the ration also supplied inadequate amounts of other amino acids. To overcome these possible deficiencies, acid-hydrolyzed fibrin prepared according to the method of Berg and Rose (6) was added to the wheat gluten basal ration at a level of 2 per cent. It will be noted that this preparation did not increase the tryptophane content of the ration because of the destruction of this amino acid during acid hydrolysis. Lysine was also added as indicated to bring the level of this amino acid to 1 per cent of the ration. As was the case with casein and gelatin, the addition of 2 per cent acid-hydrolyzed fibrin resulted in growth retardation, which was in this case partially corrected by lysine but completely so by either nicotinic acid or tryptophane (see Table III, Groups 17 through 22). When gelatin at levels of 6 or 10 per cent was added to the wheat gluten ration in addition to 2 per cent acid-hydrolyzed fibrin, the growth depression was additive and was corrected by tryptophane or nicotinic acid, whereas lysine was ineffective (Table III, Groups 23 through 28). It appears that somewhat higher levels of tryptophane would be required to overcome the combined deleterious effect of 10 per cent gelatin and 2 per cent fibrin hydrolysate.

In a simultaneous experiment acid-hydrolyzed fibrin was added at a level of 2 per cent to the 9 per cent casein ration in an attempt to supply more nearly adequate amounts of the essential amino acids lysine, histidine, and threonine, but again very poor growth was obtained. The addition of more histidine and lysine did not improve growth and not until either nicotinic acid or tryptophane was added did good growth result (see Table III, Groups 29 through 33). The further addition of either 3 or 6 per cent gelatin to this basal ration gave evidence of an additive growth-retarding action inasmuch as nicotinic acid did not correct

only 50 to 60 per cent as great as that of the synthetic substance. In addition, the synthetic compound showed some activity for strain 47904, while the isolated compound did not. Since recrystallization of the picrolonates results in compounds with identical activities for strain 34486 and no activity for strain 47904, it is concluded that a trace of an active impurity was present in the original synthetic material. The identity of the impurity is unknown.

Activity of Dimethylaminoethanol and Choline—Dimethylaminoethanol is equally active for both *cholineless* mutants in liquid media, while choline is 5 to 10 times as active for strain 47904 as for strain 34486 (2). Recrystallization of dimethylaminoethanol through the picrolonate does not significantly alter its activity for the strains. The high sensitivity to exogenous
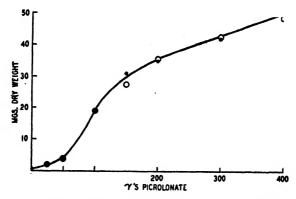
FIG. 2. Growth of strain 34486 on synthetic monomethylaminoethanol picrolonate (open circles) and on the picrolonate of the natural precursor (solid circles).

choline of strain 47904 is interpreted (2) as due to the incomplete blocking of choline synthesis, so that even on an unsupplemented medium slow growth of the mutant occurs. This view is corroborated by the results of bioassays on the Reinecke salt precipitate obtained from this strain in the course of the isolation of monomethylaminoethanol.

The reineckates obtained during the working up of 2200 gm. (wet weight) of the mutant were pooled and extracted with 50 per cent acetone. The insoluble portion was discarded. Reineckate ion was removed from the extract as silver reineckate, the solvents were removed by distillation, and the residue was extracted with alcohol. The insoluble portion was discarded and the alcohol distilled from the filtrate. The residue was dissolved in water and reprecipitated with Reinecke salt. The precipitate was washed with water and alcohol and dried, yielding 325 mg. of crude choline reineckate.

The above preparation was assayed against a standard of pure choline

reineckate by use of both *cholineless* mutants. The results of the two assays were in close agreement, 46.4 and 46.5 per cent choline reineckate, respectively. Since the sensitivities of the two strains to choline are very different, the closeness of the agreement in the two assays is strong evidence that choline is the active substance in the Reinecke precipitate. Calculated as choline chloride, the precipitate contained 49.8 mg. of choline chloride. Since a total of only 12 mg. of choline chloride was originally added to the medium in which the mutant was grown, this represents a 4-fold increase in choline content. It is concluded that strain 47904 synthesizes choline. It may be assumed that the choline isolated represents only a fraction of the total synthesized, since little, if any, phosphatidyl choline would be obtained by the procedure employed.

DISCUSSION

The foregoing results indicate that choline synthesis in the two mutants is blocked at two different stages. In strain 34486 the block precedes monomethylaminoethanol, so that the mutant cannot synthesize this intermediate but can utilize it for choline synthesis if an exogenous supply is available. If it is assumed that monomethylaminoethanol is synthesized from aminoethanol in *Neurospora*, then the block in strain 34486 occurs between these two compounds. This follows from the fact that the mutant cannot convert aminoethanol to choline (1).

In strain 47904 a partial block exists between monomethylaminoethanol and choline. This mutant can synthesize monomethylaminoethanol but is unable to convert it to choline at the normal rate; as a result, the intermediate accumulates in the cells and, eventually, in the medium. The addition of monomethylaminoethanol to the medium is without effect on the growth rate, since the mechanism for converting it to choline is already saturated. If it is assumed that dimethylaminoethanol is the intermediate between monomethylaminoethanol and choline, then the block in strain 47904 occurs between mono- and dimethylaminoethanol, since the latter is readily utilized for growth. These relations are shown in the accompanying diagram, where hypothetical intermediates are bracketed; arrows indicate the points of blocking.

$$[NH_2CH_2CH_2OH] \qquad \text{Aminoethanol}$$

Strain 34486 $\longrightarrow \qquad \downarrow$

$$CH_3NHCH_2CH_2OH \qquad \text{Monomethylaminoethanol}$$

Strain 47904 $\longrightarrow \qquad \downarrow$

$$[(CH_3)_2NCH_2CH_2OH] \qquad \text{Dimethylaminoethanol}$$

$$\downarrow$$

$$(CH_3)_3N(OH)CH_2CH_2OH \qquad \text{Choline}$$

There is some evidence that the block in strain 47904 also affects the conversion of dimethylaminoethanol to choline. When grown on agar, strain 47904 does not utilize dimethylaminoethanol as readily as does strain 34486 (2). In liquid culture, dimethylaminoethanol is equally active for both mutants; yet if its rate of conversion to choline were the same in both mutants, less should be required to produce maximum growth in strain 47904 than in strain 34486, since the block in strain 47904 is not complete. A possible explanation of these findings rests on the assumption that the same enzyme system catalyzes the methylation of both mono- and dimethylaminoethanol. Any alteration of the enzyme would be reflected in the rates of both reactions, though not necessarily to the same extent in both.

SUMMARY

As the result of single gene mutations, strains 34486 and 47904 of *Neurospora crassa* have lost the ability, possessed by the wild type, to synthesize choline. The synthesis is blocked at different stages in the two mutants, as shown by the fact that strain 47904 produces a substance which is inactive for itself but which promotes the growth of strain 34486. The substance has been isolated from cultures of strain 47904 and identified as monomethylaminoethanol. This substance is therefore a normal intermediate in the synthesis of choline by *Neurospora*. The block in strain 34486 precedes monomethylaminoethanol, while the block in strain 47904 follows it. The results throw light on the biological synthesis of choline and support the hypothesis of a simple relationship between genes and catalyzed reactions.

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THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF LABILE PHOSPHATE ESTERS

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Labile phosphate esters such as phosphocreatine, acetyl phosphate, and ribose-1-phosphate (1) have assumed great biological importance. These esters are so unstable that they are split with great rapidity by the reagents commonly used for inorganic phosphate determination. Therefore, the usual inorganic phosphate measurements in tissue extracts, etc., actually represent the sum of the inorganic phosphate and the phosphate of these labile esters. In order to determine the labile esters it has usually been necessary to perform a prior removal of inorganic phosphate by precipitation (2). By working very rapidly, Fiske and Subbarow (3) were able to measure inorganic phosphate in the presence of phosphocreatine, but, due to the speed of the reaction, this procedure has been difficult to use. The detection and determination of such esters would become very simple if it were possible to determine inorganic phosphate in their presence, since the labile phosphate could then be measured by the inorganic phosphate liberated as the result of very mild hydrolysis.

It has been found possible to establish conditions under which inorganic phosphate can readily be determined in the presence of labile esters. In addition to describing the procedure, data are presented as to the speed of hydrolysis of several of the labile esters under the prescribed conditions.

Method and Procedure

Phosphomolybdic acid is much more readily reduced to blue molybdous compounds than is molybdic acid itself. This fact is the basis of most colorimetric methods for inorganic phosphate. In the well known Fiske and Subbarow procedure (4) this reduction is effected in 0.5 N sulfuric acid by means of bisulfite with 1,4-aminonaphtholsulfonic acid as a catalyst. In the present determination the following changes from the original Fiske and Subbarow procedure are made. The pH is shifted from 0.65 to 4.0, the molybdate concentration is reduced from 0.25 per cent to 0.1 per cent, and ascorbic acid is substituted for the original mixture of bisulfite and 1,4-aminonaphtholsulfonic acid. Under these conditions the labile esters are much more stable than with the reagents prescribed by Fiske and Subbarow. The use of ascorbic acid as a reducing agent in the determination of phosphate, but at a strongly acid pH, has been previously described (5).

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The sample to be analyzed is deproteinized under conditions which will not hydrolyze the particular ester; *e.g.*, ice-cold 0.3 N (5 per cent) trichloroacetic acid or 0.3 N (3 per cent) perchloric acid, or, particularly with very labile esters, saturated ammonium sulfate which is 0.1 N in acetic acid and 0.025 N in sodium acetate (pH 4). If either of the acid precipitants is used, the extracts are rapidly brought to pH 4 to 4.2 by adding 4 volumes of 0.1 N sodium acetate. Most of the labile esters are reasonably stable at this pH. The extracts are diluted with acetate buffer of pH 4 (0.1 N acetic acid, 0.025 N sodium acetate) until the inorganic phosphorus is 0.015 to 0.1 mM (0.05 to 0.3 mg. per cent of P). Ammonium sulfate extracts should be diluted at least 5-fold. To each volume of extract is added 0.1 volume of 1 per cent ascorbic acid and 0.1 volume of 1 per cent ammonium molybdate in 0.05 N sulfuric acid. Readings are made at 5 and again at 10 minutes after the molybdate addition at a wave-length of 700 mμ. (Any wave-length between 650 and 950 mμ is satisfactory.) Simultaneous readings are made on a 0.05 mM standard and a blank, both of the same composition, as far as possible, as the unknown. If a difference is observed in the unknown readings at 5 and at 10 minutes compared to the standard, the values are extrapolated to zero time. The ascorbic acid and molybdate may be combined before addition but must then be used within 15 minutes.

In the presence of certain tissue extracts, the reaction is delayed, in which case an internal standard must be used. A standard amount of inorganic phosphate is added to a duplicate tissue aliquot, and values of the unknown are calculated from the difference between the readings of the unknown and of the unknown with added phosphate. The inhibitory effect may be partially overcome by dilution. For example, in order to avoid undue inhibition, brain and muscle extracts should be diluted to a volume 150 to 250 times that of the tissue, and liver extracts to a volume 300 to 500 times that of the original liver. In addition, the molybdate concentration may be increased to 0.15 per cent; *i.e.*, 0.1 volume of 1.5 per cent ammonium molybdate in 0.05 N sulfuric acid is added in place of the 1 per cent solution. This results in an acceleration of color development. In studying isolated enzyme systems, this problem of inhibition is ordinarily not encountered.

The ascorbic acid concentration may be increased, if necessary, to accelerate the reaction, but with final concentrations greater than 0.2 to 0.3 per cent the readings of the standard increase unduly with time. The final pH may be varied, if desired, between 3.5 and 4.2.

DISCUSSION

The following observations suggested the possibility that inorganic phosphate might be determined under conditions which would be less likely to

hydrolyze labile phosphate esters.   As the acidity of a mixture of molybdic acid and reducing agent is progressively changed, an interesting phenomenon occurs (Fig. 1).   Molybdic acid itself is reduced at intermediate pH levels, resulting in a large and progressive blank.   With further increase in pH, molybdate is no longer reduced; yet phosphomolybdate may still undergo reduction.   Thus, with aminonaphtholsulfonic acid and bisulfite as reducing agents, a large reagent blank is encountered between pH 0.8 and 1.8, and with the stronger ascorbic acid reducing agent, the reagent blank becomes very large between pH 0.9 and 2.8 (Fig. 1).   Phosphomolybdate continues to be reduced at a useful rate as far as pH 3.2 with bisulfite, and as far as pH 4.6 with ascorbic acid.   Therefore, with the Fiske and
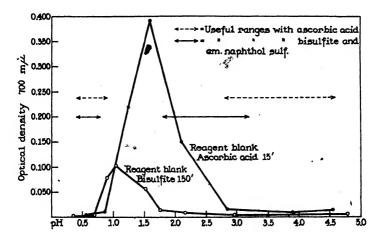
FIG. 1. Reagent blank values at different pH levels and useful pH ranges with (a) ascorbic acid and (b) bisulfite-aminonaphtholsulfonic acid as reducing agents.

Subbarow reagents, phosphate may be measured between pH 0.4 to 0.7 and pH 1.8 to 3.2, and with ascorbic acid between pH 0.4 to 0.9 and pH 2.8 to 4.6 but not at intermediate pH levels.   Since acidity accelerates the splitting of labile phosphate esters, ascorbic acid with its more nearly neutral effective range was chosen.   Actually, the less acid pH range with ascorbic acid is limited to pH 3.5 to 4.5, since between pH 2.8 and 3.5 color continues to develop unduly with time.

Effect of Ascorbic Acid and Molybdate Concentrations on Color Development—In general, it has been found that the higher the ascorbic acid concentration the more rapid is the reduction of phosphomolybdic acid.   If the ascorbic acid concentration is too great, the standard continues to develop color with time.   The concentration recommended, 0.1 per cent, is a satisfactory compromise.   Decreasing the molybdate concentration

tends to slow the rate of color development. Reduction in the concentration of molybdate is desirable in order to decrease its catalytic effect in splitting phosphocreatine or acetyl phosphate; however, the concentration cannot be reduced much below 0.05 per cent without inordinate decrease in the rate of color development. Indeed, if the concentration is much below this figure full color is never developed. It is worth noting that if ascorbic acid stands with the weak molybdate reagent at pH 4.0 before phosphate is added the reagent becomes incapable of developing color rapidly and the reaction does not proceed to completion. The mechanism of this effect is obscure; it is not due to destruction of ascorbic acid. It is, therefore, essential that the ascorbic acid and molybdate are not mixed together until just before use.

The reaction is slower the higher the pH. With 0.25 per cent molybdate the reaction is sufficiently rapid up to pH 4.6. With 0.05 per cent molybdate pH 4.2 is about the practical pH limit. Since change of hydrogen ion concentration has less effect in this pH range on the splitting of phosphate esters than the molybdate concentration, it is desirable to work in the more acid region with the lowest permissible molybdate concentration. The reaction is accelerated by raising the temperature; for example, the reaction is essentially complete in 2 minutes at 35° and 6 minutes at 25°. At 0° almost no color is produced.

Disturbing Substances—The following concentrations of added substances were found not to cause disturbing change in color development: sodium acetate 0.5 M, sodium sulfate 0.3 M, potassium nitrate 0.3 M, ammonium sulfate 0.2 M, sodium chloride 0.15 M, sodium perchlorate 0.15 M, sodium trichloroacetate 0.15 M, sodium fluoride 0.13 N, glycine 0.02 M, sodium oxalate or citrate 0.05 M, sodium nitrite 0.004 M, sodium tungstate 0.004 M, and ferric chloride 0.003 M. With larger amounts of many of these substances the color development is delayed. This delay can often be overcome by increasing the ascorbic acid concentration to 0.2 per cent. Unfortunately, color develops with both arsenate and silicate. Not over 2 mg. per cent of arsenate or 0.5 mg. per cent of silicate is tolerable. It is, therefore, necessary to avoid reagents such as sodium hydroxide, which may contain silicate. In neutralizing acids, sodium acetate is desirable, since it is not likely to contain silicate and it buffers the reaction at an appropriate pH.

Proportionality and Reproducibility—The optical density of solutions with different amounts of phosphate is linear from 10 to 75×10^{-6} mole per liter (0.03 to 0.25 mg. per cent of P). This is equivalent to extinctions (optical densities with a 1 cm. light path) from 0.05 to 0.35 at 700 mμ. The reproducibility in the Beckman spectrophotometer is ±1 or 2 per cent over this range, depending on the concentration. The absorption is essentially the same from 700 to 900 mμ, with a maximum at 860 mμ.

Effect on Labile Esters—Figs. 2 to 4 illustrate the hydrolytic rates for various phosphate esters under different conditions of pH and molybdate concentration. The rates were measured at room temperature without any attempt at exact temperature control. All of the rates are, of course, influenced somewhat by the temperature. The accelerating effect of molybdate on the splitting of phosphocreatine and acetyl phosphate has been previously observed. (2, 4). Phosphocreatine (Fig. 2) is split under the conditions described by Fiske and Subbarow for inorganic phosphate determination with a half time of about 4 minutes. At pH 4, with 0.25
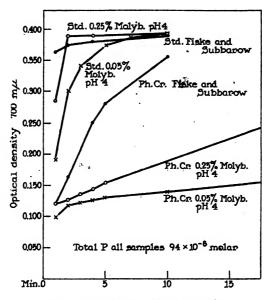
FIG. 2. Splitting of phosphocreatine

per cent molybdate, the half time is nearly 20 minutes, and with 0.05 per cent molybdate at pH 4.0 the half time is about 70 minutes. With acetyl phosphate (Fig. 3) in the strong acid reagent the half time of hydrolysis is 30 or 40 seconds, at pH 4.5 with 0.25 per cent molybdate it is 12 minutes, and at pH 4 with 0.05 per cent molybdate it is approximately 90 minutes. It would appear that the change in pH affects both esters to about the same degree but that molybdate has a greater relative effect on acetyl phosphate than on phosphocreatine.

Ribose-1-phosphate (Fig. 4) has a half time of about 2.5 minutes in the Fiske and Subbarow reagents. The hydrolysis is much slower at pH 4.0. The half time with 0.25 per cent molybdate appears to be over 3 hours. Thus, the change in stability with pH is relatively greater for ribose-1-
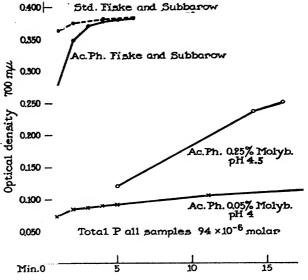
FIG. 3. Acetyl phosphate splitting
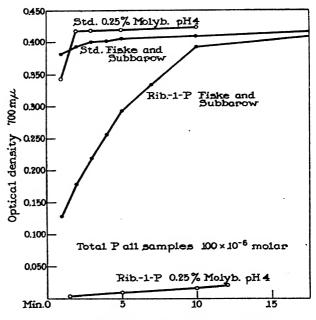
FIG. 4. Ribose-1-phosphate splitting

phosphate than for phosphocreatine or acetyl phosphate. Dr. H. M. Kalckar, in this laboratory, has observed that molybdic acid does not accelerate the splitting of ribose-1-phosphate.

Representative Tissue Analyses—Rat skeletal muscle and brain were analyzed for inorganic phosphorus with both the Fiske and Subbarow reagent and the ascorbic acid reagent at pH 4.0. The extracts used were prepared with ice-cold ammonium sulfate at pH 4.0 from tissues frozen *in situ* with petroleum ether chilled with solid carbon dioxide. The final
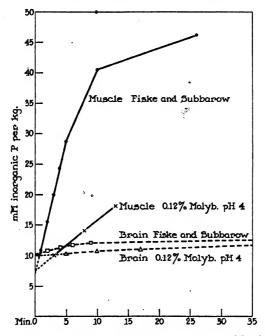
FIG. 5. Inorganic phosphate in muscle and brain

reagent was 0.125 per cent in ammonium molybdate and the final tissue dilution was about 500-fold. With both reagents approximately the same extrapolated values are obtained for inorganic phosphate. This indicates the absence of significant amounts of acetyl phosphate (Fig. 5). With the ascorbic acid reagent the change in the inorganic phosphate concentration is slow enough to make it easy to obtain the initial inorganic phosphate value.

SUMMARY

1. A procedure is described for the measurement of inorganic phosphorus in the presence of labile phosphate esters. The reaction is based on the reduction of phosphomolybdate by ascorbic acid at pH 4.0.

2. Under the reaction conditions the splitting of phosphocreatine, acetyl phosphate, and ribose-1-phosphate is decreased to 5 per cent or less of the rate observed in the presence of the Fiske and Subbarow reagents. This makes possible a simple determination of the concentration of such labile esters.

3. There do not appear to be significant amounts of acetyl phosphate in skeletal muscle or brain.

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A HOMOMOLECULAR SERUM PROTEIN WITH ANOMALOUS SOLUBILITIES

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A flocculent material, shown to be a protein, spontaneously precipitated from the serum of a patient, J. P., at the University of Minnesota Hospitals during the course of routine blood studies *in vitro*.

The conditions under which a protein spontaneously precipitates from serum are uncommon. Records of such conditions are even less frequently met with; only some half dozen reports of such cases have been made (1–6). Three of these reports were based upon cases in which the serums were obtained from patients having multiple myeloma. (The precipitated proteins were not of the Bence-Jones type.) The other three reports described the flocculation of protein from serum of humans suffering either from chronic rheumatic infectious arthritis and spondylitis, or from liver disease, and from dogs infected with kala-azar.

Because spontaneous precipitation of protein from serum is a rare occurrence, it was considered worth while to undertake the isolation and determination of the physical and chemical characteristics of the protein that precipitated from the serum of the patient J. P.

Since the clinical aspects of this exceptional case will be reported elsewhere,[1] suffice it to say that the patient was a 58 year-old white male with acro purpura of 9 years duration, chronic glomerulonephritis, and congestive heart failure.

Isolation

A 320 ml. sample of the patient's blood was allowed to clot at 37° and 220 ml. of serum were decanted. Upon cooling the serum to 2°, a flocculent precipitate formed. This was centrifuged at 0° and the supernatant fluid poured off. To the precipitate were added 120 ml. of 0.9 per cent sodium chloride. By warming to 37°, all but a minor portion (Fraction A) of the substance dissolved. The solution was centrifuged at 37° with the resulting removal of the few red cells from the fraction precipitated in the cold. This process of purification was repeated three times. In each case a small amount of material insoluble at 37° was formed (Fractions B, C, and D).[2]

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[1] To be published by Dr. C. J. Watson.

[2] It should be noted that an increased volume of saline did not affect the solution of the insoluble portions.

When the purified solution was kept overnight at a temperature of 2°, precipitation occurred. The solution was centrifuged at 0°, and 120 ml. of water were added to the precipitate. All but a negligible portion dissolved at 37°. Precipitation was again effected under the same conditions; *viz.*, by cooling at 2° for 12 hours.

The addition of 120 ml. of water to the precipitate resulted in easy solution at room temperature. At this point it was discovered that further cooling of the solution at 2°, even over a period of several weeks, did not bring about precipitation. By drying aliquot parts of solution at 100° for 3 hours and weighing the residue, it was found that the solution, totaling 180 ml., contained 0.93 per cent of protein.

Properties

The solution gave very strong biuret, xanthoproteic, Millon, and Hopkins-Cole reactions. A quantitative colorimetric biuret analysis, with purified horse γ-pseudoglobulin as a standard, showed that the dry residue of the solution was entirely protein. The Molisch, Bial, phosphorus, and cholesterol tests were negative. The protein was heat-coagulable. Some of the properties of the protein are listed in Table I.

Absorption Spectrum—The ultraviolet absorption spectrum of the protein was determined with a Beckman quartz spectrophotometer with 1 cm. cuvettes at room temperature. The absorption curve, Fig. 1, resembles that of the γ-globulins.[3] The solution had a pH of 5.7. Maximum absorption was at 2780 A and minimum absorption at 2505 A. Absorption is expressed as $E_{1\,cm.}^{1\%}$. This refers to the optical density of a 1 per cent solution in a thickness of 1 cm.

Solubility—Quantitative determinations of the supernatant solutions in the solubility measurements were carried out with a Beckman quartz spectrophotometer at 2800 A after dilution to protein concentrations of 5 to 50 mg. per 100 ml. Known concentrations of the protein were used as standards. It was shown by Smith (7) that at 2800 A solutions of pseudoglobulins obey the Beer-Lambert law over a wide range of concentrations. Fig. 4 shows that the protein under consideration obeyed this law for the concentration range employed in this work. Greenberg[4] has shown that salts in low concentration do not affect the extinction values at 2800 A for bovine γ-pseudoglobulin and that high salt concentrations have only a small effect.

Equal volumes of varying concentrations of sodium chloride or ammonium sulfate solutions were added with shaking to equal volumes of the 0.93 per cent protein solution. Thus the final volumes and the total pro-

[3] Unpublished data.
[4] Unpublished data.

tein concentrations were the same in all cases.   Equilibration was attained
by allowing the solutions to stand 24 hours at room temperature with
occasional shaking.   The supernatant solutions were analyzed spectro-
photometrically after centrifugation.

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TABLE I

Properties of Protein

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Isoionic point, pH	5.7
Kjeldahl nitrogen, %	15.7
Mol. wt.	190,000
Viscosity (0.93% solution), η/η_0	1.130
Specific rotation, $[\alpha]_D^{26}$	-66.3
Mobility (pH 4.7), *cm. per sec. for 1 volt per cm.*	$+3.38 \times 10^{-5}$
Ultraviolet absorption (pH 5.7), $E_{1cm.}^{1\%}$	13.3 (2780 A; maximum)
	5.0 (2505 " minimum)
Salting-out constants, K_s	
$(NH_4)_2SO_4$	2
NaCl	25–35
Heat of solution (0.5% NaCl), $\Delta \bar{H}$, *calories*	
1–20°	13,300
26–36°	36,500
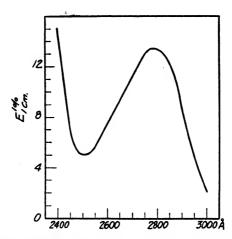
FIG. 1. Absorption spectrum of an aqueous protein solution

The solubility of this protein in solutions of varying ionic strength was
unique (Fig. 2).   Although there was not sufficient protein available to
determine its exact solubility in water, the substance appeared to be
moderately soluble.[5]   The protein was insoluble in 0.04 to 0.16 M (0.23 to

[5] The dilute sodium chloride solutions of the protein (below 0.04 M) were apparently
in a state of true equilibrium.   However, at the present time no definite conclusion

0.92 per cent) sodium chloride at room temperature. Upon increase of salt concentration, the protein went into solution. Whereas saturation with sodium chloride did not precipitate the protein, a salt concentration of 1.3 to 1.4 M (34 per cent) ammonium sulfate resulted in precipitation.

With approximately 0.06 per cent sodium chloride, precipitation commenced at 2°, compared with 0.23 per cent sodium chloride required for precipitation at room temperature. In 0.5 per cent sodium chloride at room temperature the protein was soluble on either side of its isoelectric point. No precipitate formed in a 0.1 per cent solution of the protein in 86 per cent ethyl alcohol.

When the protein was precipitated from solution with 0.5 saturated ammonium sulfate or 22 per cent sodium sulfate, it dissolved in distilled water but did not precipitate out of dilute sodium chloride solution.
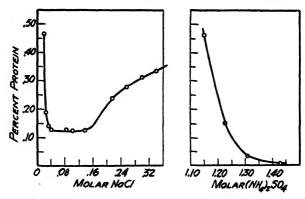
FIG. 2. Protein solubility in solutions of varying salt concentrations at 26°

The fractions that did not redissolve in the purification procedure dissolved in physiological saline solution warmed to 42°. Precipitation occurred on cooling the solutions. When the precipitates were separated from the solutions and resuspended in fresh saline, they dissolved at 37°, as had the original material.

The relation of solubility to temperature (Fig. 3) was determined in a manner similar to that employed in the salt solubility studies except that the final sodium chloride concentration in every case was 0.5 per cent. Temperatures of 2°, 19.7°, 26°, 33°, and 36° were used. Because of the greatly increased solubility at the last two temperatures and the scarcity of

can be made concerning the equilibrium state because sufficient protein was not available for further study. It can be seen that if an equilibrium state were not present, the increased solubility of the protein could be explained by assuming that the protein molecule carries an appreciable electrical charge and remains in solution as a result of the repulsive forces between the charged protein molecules.

the protein, the values at these points were determined by suspending known weights of protein in the sodium chloride solution and slowly warming the mixture in a water bath until complete solution occurred. Solubilities at the lower temperatures were determined after 24 hour equilibration, centrifugation at the same temperature, and analysis of the supernatant solutions.

As is shown in Fig. 3, a 0.93 per cent solution of the protein in distilled water showed no precipitate at 2°. However, in 0.085 M (0.5 per cent) sodium chloride at that temperature, the protein was almost completely
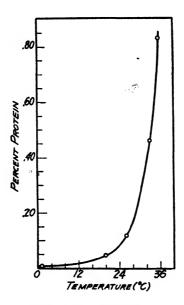
FIG. 3. Relation between solubility and temperature in 0.5 per cent NaCl solution

insoluble. As the temperature was increased, a marked increase in solubility occurred.

Salting-Out Constants—The relationship between the solubility and ionic strength can be represented by the equation ((8) p. 604), $\log S = B - K_s \mu$, where S = the solubility of the protein in gm. per liter, μ = ionic strength, K_s = the slope of the curve and for a given protein and salt is independent of temperature and pH, B = the logarithm of the solubility of the protein in solutions of 0 ionic strength and is dependent on temperature and pH. For the protein studied here, K_s for ammonium sulfate solutions = 2.0 and for dilute sodium chloride solutions is somewhere between 25 and 35. The value of approximately 30 for K_s in dilute sodium chloride solutions is much greater than any previously calculated salting-out con-

stant (8).   However, because conditions necessary for equilibrium may not have been satisfied, it is possible that the K_s calculated for the protein in very dilute sodium chloride solutions may not be a true salting-out constant.

Heat of Solution—The heat of solution of a protein is usually calculated ((8) p. 587) by determining the solubility, S, of the protein in a particular solvent at various temperatures and then using the formula[6]

$$\overline{\Delta H} \quad \frac{R \ln \dfrac{S_2}{S_1}}{\dfrac{1}{T_1} \quad \dfrac{1}{T_2}}$$

Phase Rule Studies—These studies, carried out by techniques similar to those used in the investigation of solubility properties, gave evidence that the protein solution might consist of a single component (Fig. 4).   For confirmation, the experiment was performed twice.
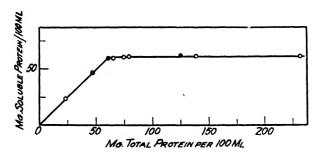
FIG. 4. Phase rule studies in 0.5 per cent NaCl at 19.8° (two experiments)

To varying concentrations of protein solutions were added equal volumes of 0.9 per cent sodium chloride solutions, so that the final total volume and concentration of sodium chloride were the same.   Equilibration was brought about by allowing the mixtures to stand, with occasional shaking, for 24 hours in one case and 48 hours in the other.   The temperature was 19.7° for both experiments.   Centrifugation was carried out at the same temperature.

Isoionic Point—The pH of a salt-free 0.93 per cent solution[7] of the protein as determined by the glass electrode was 5.70.   According to Cannan (9) this value should be the isoionic point.

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[6] Strictly speaking, this is not the heat of solution.   If this procedure were used, one actually would obtain the value of the average heat absorbed over the temperature range T_1 to T_2 when 1 mole of protein dissolves in a saturated solution.

[7] This so called "salt-free solution" could have a sodium chloride concentration as great as 0.015 M.   Nevertheless, this low salt concentration could not affect the isoionic point (9).

Total Nitrogen—A small portion of the purified protein solution was evaporated to dryness. The residue was heated at 100° for 3 hours. Kjeldahl determinations of the total nitrogen content were made on the dried sample by a modification of the Cavett method (10). The nitrogen content was 15.7 per cent.

Osmotic Pressure—With the apparatus of Keys and Taylor (11), two determinations at 2° of the 0.93 per cent protein solution against water showed osmotic activity that corresponded to average molecular weights of 180,000 and 210,000. These values are to be considered only rough estimates of the molecular weight.

Viscosity—An Ostwald viscometer was used in a water bath at 26°. Six determinations were made on 8 ml. portions of both protein solutions and distilled water. The concentration of protein was 0.93 per cent. Its viscosity compared to water was 1.130.
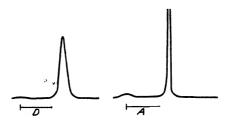
Fig. 5. Electrophoretic pattern; pH 4.7; 0.1 ionic strength

Optical Rotation—The optical rotation was carried out on the 0.93 per cent protein solution at 26° with a 2.00 dm. tube and a sodium lamp; $[\alpha]_D^{26} = -66.3°$.

Electrophoretic Pattern and Mobility—The protein migrated with a single symmetrically shaped boundary, as is shown in Fig. 5. The electrophoretic mobility was determined with a 0.5 per cent protein solution buffered to pH 4.7 with acetic acid-sodium acetate at 0.1 ionic strength. In 9000 seconds the ascending boundary moved a distance of 2.27 cm. The mobility was 3.38×10^{-5} cm. per second for 1 volt per cm.

Stability—A saturated aqueous solution of the protein appeared to have its original solubility characteristics even after standing at room temperature for 3 months.

Crystalline Structure—A 1 ml. solution of 0.5 per cent chloride and 0.465 per cent protein was warmed to 35° and placed in a thermos flask containing water at this temperature. After the flask had remained at room temperature for 5 days, particles, which appeared to be doubly refractive, formed. Although the crystalline structure could not be made out definitely, it

appeared to resemble the structure of the particles studied by Holmberg and Gronwall (4).

Wassermann Reaction—Because the serum of Holmberg and Gronwall's patient had given an atypical Wassermann reaction, this test was performed on the protein and serum of J. P. Both the serum and a 0.93 per cent solution of the protein were negative.

<div align="center">DISCUSSION</div>

The protein which spontaneously precipitated from the serum of the patient J. P. had several unusual properties. With respect to solubility, ultraviolet absorption spectrum,[3] and nitrogen content (12), it resembled the γ-globulins. However, this protein, unlike some γ-globulins, was soluble in water but insoluble in dilute salt solutions. Its molecular weight (13) and viscosity (14) also seemed greater than those for γ-globulins.

The protein solution finally obtained appeared to be homomolecular. As yet no serum globulin preparation has satisfied all of the criteria for homogeneity. However, in consideration of the unique properties of this protein which made possible the previously described method of isolation, electrophoretic pattern, and phase rule studies, it seems probable that the solution contained a single protein solute.

From the available data on similar proteins studied by workers in recent years, a comparison of the properties of these substances was made in Table II. One characteristic common to all of the proteins reported was spontaneous precipitation from serum. Unfortunately, detailed investigations of the proteins discovered earlier by other workers were not carried out, thus limiting the scope of the comparative analysis made at the present time.

The unique character of the protein from the serum of J. P. resulted in part from the unusual solubility properties, as is shown in Fig. 2; *viz.*, the protein was soluble in water, insoluble in dilute solutions of electrolytes, soluble in more concentrated solutions of electrolytes, and insoluble in 23 per cent sodium sulfate or 33 per cent ammonium sulfate. Apparently no investigator carrying out similar studies attempted to free the protein solution completely from electrolytes and then to determine its solubility in water. However, von Bonsdorff, Groth, and Packalen stated that the protein studied by them was insoluble in physiological salt solution and distilled water but that "the wash water, after repeated washings...still yielded a relatively strong protein reaction." This probably means that their protein was also soluble in water.

It is important to remember that once the protein from the serum of J. P. was treated with ammonium or sodium sulfate it behaved thereafter as a pseudoglobulin, *i.e.*, it was soluble in water and in dilute salt solutions, thus

TABLE II

Comparison of Serum Proteins

Observer	Carbohydrate	Phosphorus	Crystalline structure	Mol. wt.	Wassermann reaction	Stability	pH, maximum insolubility	Mobility, pH 4.7 cm. per sec. for 1 volt per cm.
Lerner and Greenberg	0	0	Cuboidal (?)	190,000	0	Very stable	5.7–6.2	3.38 × 10⁻⁶
Holmberg and Gronwall	Positive	0	Cuboidal		+	Apparently stable	10.7	3.4 × 10⁻⁵
Stein and Wertheimer	0	0	Rhombic hemi-morphous	200,000	"	Very labile	5.7–6.2	
Von Bonsdorff, Groth, and Packalen						" stable		
Wintrobe and Buell		+			"	Apparently stable		
Bing				No data available				
Atlas, Cardon, and Bunata				" "				

proving that the addition of these salts was responsible for the change in solubility. Consequently, proteins of similar nature should be studied before precipitation with ammonium or sodium sulfate.

Stein and Wertheimer observed that once their protein was treated with ammonium or sodium sulfate it lost its ability to precipitate from cooled dilute salt solutions; *i.e.*, the protein became soluble in water and dilute salt solutions. The same workers stated that their protein was "denatured." These solubility changes were unusual, for ordinarily when treated with any reagent, proteins become less soluble. A question arises concerning the solubility properties of the well established serum globulins. One might well ask whether the serum pseudoglobulins would be insoluble in very dilute salt solutions if they were prepared by a non-precipitating method, such as the Tiselius method.

Holmberg and Gronwall stated that the protein they studied precipitated when the blood samples were exposed to air, but that when the samples were kept in an air-tight container no flocculation occurred. This indicates that their protein would probably precipitate if carbon dioxide were released from the blood and the pH of the solution increased. This protein spontaneously precipitated at room temperature, its solubility appearing to depend more on the pH than on the temperature of the solution.

The protein studied by Wintrobe and Buell likewise precipitated at room temperature. Although, in the two cases last mentioned, the proteins precipitated at room temperature, in most investigations it was necessary to cool the serums before precipitation took place.

It is a well known fact that pathological serums may contain unusual proteins. What is not so well known is that the symptoms of a disease may be directly related to the physical properties of an unusual protein,[1] a protein which in the case of the patient J. P. spontaneously precipitated from cooled serum.

SUMMARY

1. A protein spontaneously precipitated from cooled blood of a patient having acro purpura, chronic glomerulonephritis, and congestive heart failure.

2. The protein was isolated in pure form by separating serum from blood clotted at 37° and then cooling the serum. The precipitate from the cooled serum was redissolved and reprecipitated from physiological saline solutions several times.

3. The increased solubility of this protein in 0.2 M sodium chloride and its salting-out curve with ammonium sulfate at room temperature were characteristic of γ-euglobulins. However, this protein was also soluble in distilled water.

4. The method of isolation, the electrophoretic pattern, and the phase rule studies indicated that the purified protein preparation probably was homogeneous.

5. The protein was compared with similar substances described in reports of other investigations and was shown to have some unique characteristics. One property common to all of the proteins compared was their spontaneous precipitation from serum (usually cooled).

We wish to express our sincere appreciation to Dr. C. J. Watson for making possible this study through his keen interest in the problem. We should like to thank Dr. D. R. Briggs for carrying out the Tiselius studies and Dr. C. P. Barnum for the use of his laboratory in which the work was accomplished.

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SYNTHESIS OF CHOLESTEROL IN SURVIVING LIVER*

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The surviving tissue slice technique has become one of the principal methods for studying the chemical reactions of the living cell. The tissue slice experiment in general consists in the addition to the slices suspended in an aqueous buffer of some suspected metabolite and measurement of the rate of change of concentration of the compound or of some chemically related compound. The tissue slice itself does not seem to enter into the chemical reactions, behaving more as if it were an inert framework to which enzymes and their coenzymes are attached. It has never been possible for example to demonstrate the synthesis of any structural component of the tissue slice.[1] Further, this technique is limited to the study of compounds soluble in the buffer. The demonstration of biological reactions in surviving tissues has been most successful with freely diffusible small molecular substances as substrates. The majority of the processes studied *in vitro* have been of the nature of degradative reactions. When synthesis of carbon-carbon linkages could be demonstrated, the number of carbon atoms involved has been small.

The rates at which tissue constituents are regenerated by synthesis in the intact animal vary widely. It is conceivable that a biochemical process which is slow *in vivo* may escape detection *in vitro*, because too little of the reaction product accumulates during the relatively short period of tissue survival. Further, even if a reaction is sufficiently rapid, its occurrence may be obscured if the reaction product is metabolized faster or at the same rate at which it is formed. In such a case the concentration of the metabolite will decrease or remain unchanged during the experiment, precluding detection of the process by quantitative analysis. However, if a substrate labeled by an isotopic element is employed, appearance of the isotope in the reaction product will be sufficient proof for utilization of the substrate

[1] An apparent exception is the demonstration of the formation of radioactive lipids after incubation of liver slices with radioactive phosphate (1). Other such examples might be found but in these cases we are apparently dealing with small changes on the periphery of a large molecule. These reactions do not involve the formation of carbon to carbon linkages.

irrespective of whether or not the quantity of the product underwent a change.

Synthesis of cholesterol in the intact animal has been demonstrated by enriching the body fluids with heavy water (2) and by administration of labeled acetic acid (3). In both cases isotopic cholesterol is formed. The rate at which isotope is incorporated into the cholesterol molecule indicated that regeneration of cholesterol is slow compared to that of other tissue constituents such as glycogen, fatty acids, and amino acids. It can be estimated that in the liver of the rat half of the cholesterol molecules are replaced by newly formed cholesterol in 5 to 10 days. If cholesterol were synthesized as rapidly in surviving liver as in the living rat, the cholesterol concentration could not change by more than a few per cent during the period of incubation. The experiments of Sperry et al. (4) and those of Moulder and Evans[2] indicate that on incubation of rat liver slices either without added substrate or in the presence of acetate no significant change occurs in the amount of cholesterol.

We have followed cholesterol synthesis in surviving rat liver by adding either heavy water or labeled acetic acid to the buffer fluid. Deuterio cholesterol was formed in the presence of either D_2O or deuterio acetic acid. Incubation with acetic acid containing C^{13} resulted in the formation of cholesterol containing heavy carbon. Since acetic acid has been shown to participate in the synthesis of the higher fatty acids (5), we have also isolated the fatty acids from the incubated slices. They contained small but significant concentrations of deuterium.

EXPERIMENTAL

Deuterio acetic acid was prepared as described previously (3) and converted into the sodium salt. It contained 59.0 atom per cent excess deuterium. The preparation of acetic acid containing 77 atom per cent excess deuterium in the methyl group and 19.6 atom per cent excess C^{13} in the carboxyl group has been described previously (5).

Incubation Experiments—Slices were prepared from the livers of young rats (120 to 230 gm.). The tissue was suspended in Krebs' phosphate buffer, pH 7.3, and incubated after addition of either labeled acetic acid or D_2O for 3 hours at 38°. For each experiment 2 to 3 gm. of liver tissue (wet weight) were used. The volume of buffer varied from 10 to 50 ml.

Isolation of Cholesterol and Fatty Acids—At the end of the incubation period an equal volume of ethanol and sufficient solid KOH to make a 10 per cent solution were added to the flasks. The mixture was heated under a reflux for 5 hours and separated into saponifiable and unsaponifiable fractions. From the unsaponifiable fraction cholesterol was precipitated

[2] Private communication from Dr. E. A. Evans, Jr.

as the digitonide. After removal of the unsaponifiable fraction the alkaline hydrolysate was acidified and the fatty acids extracted with petroleum ether. From 2 to 3 gm. of incubated liver slices there were isolated 4 to 6 mg. of cholesterol (calculated from the weight of the digitonides) and 50 to 70 mg. of fatty acids. Deuterium analyses were carried out by an unpublished micromethod. In most cases the deuterium content of cholesterol was obtained by analysis of cholesterol digitonide and multiplying the value by 3.

Incubation of Liver Slices with Na Acetate Containing Deuterium and C^{13}. *Experiment A*—2.0 gm. of liver slices from rats weighing 220 to 230 gm. were incubated aerobically in 20 ml. of phosphate buffer to which had been added 10 mg. of Na acetate containing 77 atom per cent excess deuterium in the methyl group and 19.6 atom per cent excess C^{13} in the carboxyl group. The cholesterol digitonide (20 mg.) obtained was decomposed in the usual fashion (6) and the cholesterol recrystallized from 0.5 ml. of acetone. 2.7 mg., m.p. 146–147°, were obtained, which contained 0.11 atom per cent excess deuterium and 0.038 atom per cent excess C^{13}.

Experiment B—3.0 gm. of liver slices from rats (120 gm.) were incubated aerobically in 10 ml. of phosphate buffer to which had been added 20 mg. of Na acetate containing 9.8 atom per cent excess C^{13} and 77.0 atom per cent excess D. 26.4 mg. of cholesterol digitonide yielded 3.5 mg. of cholesterol, m.p. 145–147°. It contained 1.03 atom per cent excess deuterium and 0.40 atom per cent excess C^{13}.

Incubation of Organs Other Than Liver—Slices from spleen, kidney, testes, and intestine of the rat were incubated aerobically in the presence of 20 mg. of deuterio acetate containing 59.0 atom per cent excess deuterium. The cholesterol digitonides isolated from these organs contained in no case more than 0.015 atom per cent excess deuterium.

DISCUSSION

In all experiments the appearance of the isotopic label in cholesterol is taken as proof for the occurrence of synthesis. The observed process must represent synthesis of the carbon skeleton. Not only is deuterio cholesterol formed in the presence of heavy water, but the cholesterol isolated after incubation with acetic acid containing deuterium in the methyl group and C^{13} in the carboxyl group contained both deuterium and C^{13}.

With the aid of isotopically labeled substances reactions proceeding at very slow rates become readily detectable. For example if in one of our present experiments the cholesterol isolated after incubation with deuterio acetate (59 atom per cent excess deuterium) contains the significant concentration of 0.10 per cent D, a quantity of cholesterol corresponding to 0.17 per cent of that originally present must have been newly formed. As the

tissue slices used in our experiments contained about 5 mg. of cholesterol, the above figure corresponds to a synthesis of 8 to 10 γ.

From the data presented in Table I it is clear that aerobic conditions are necessary for cholesterol synthesis to occur. It is noteworthy that all *in vitro* reactions so far studied with acetic acid as a substrate seem to require the presence of oxygen even if oxygen does not enter into the equation of the reaction concerned. This is the case in the formation of acetylsulfanilamide, acetylcholine, acetoacetic acid, and citric acid.[3] Addition of glucose was without effect on cholesterol formation, suggesting that intermediates of glucose metabolism, if they participate in the process, are present in the slice in sufficient quantities.

Formation of isotopic cholesterol was not observed in disintegrated liver tissue such as homogenized liver or hash prepared in the Waring blendor (see Table II). It is not surprising that systems in which the structure is

TABLE I

Isotope Concentration in Cholesterol and Fatty Acids Isolated from Rat Liver Slices Incubated with 150 Mg. of Deuterio Acetate Containing 59.0 Atom Per Cent Excess Deuterium

Time of incubation 3 hours; buffer volume 50 ml.

	Atom per cent excess deuterium	
	Cholesterol	Fatty acids
Aerobic and glucose..........................	0.224	0.015
"	0.279	0.017
Nitrogen, glucose............................	0.003	0.005
"	0.003	0.005

destroyed cannot synthesize a substance of such complexity as cholesterol, for it is probable that many enzyme systems must cooperate in the synthesis. Since at least some of the intermediates must be almost as non-diffusible as the end-product, the enzyme systems which are involved in the formation of the carbon-carbon linkages as well as those which supply the energy for these reactions must be suitably organized in space.

When the quantities of deuterio acetic acid added to the buffer solution were varied from 10 to 150 mg. per 2 gm. of liver slice, no significant change in the isotope concentration of cholesterol took place (Table III). Since under our conditions the quantities of acetate required must have been of the order of 0.5 to 1.0 mg., the substrate added must have been in excess.

[3] Anaerobic acetylation of choline (7) and sulfanilamide (8) by acetic acid in the presence of adenosine triphosphate has recently been reported. The ATP presumably supplies the energy required for this coupling.

TABLE II

Isotope Concentration in Cholesterol Isolated after Aerobic Incubation of Liver Preparations

	Acetate added (59 atom per cent D)	Addition	Cholesterol, atom per cent excess deuterium
	mg.		
Homogenate	150	100 mg. glucose	0.006
"	150	40 " ATP* + 85 mg. NaF	0.004
Pulp (Waring blendor)	150	100 " glucose	0.009
" " "	50	35 " NaF + 40 mg. ATP†	0.002
" . " "	50	40 " ATP*	0.008

* ATP = adenosine triphosphate.
† Added in three portions.

TABLE III

Isotope Concentration in Cholesterol Isolated from Liver Slices Incubated Aerobically with Varying Amounts of Deuterio Acetate (59.0 Atom Per Cent Excess Deuterium)

Buffer volume 50 ml.

Deuterio acetate	Cholesterol, atom per cent excess deuterium
mg. per flask	
150	0.063
50	0.072
25	0.075
10	0.072

TABLE IV

Isotope Concentrations in Cholesterol from Liver Slices Incubated Aerobically in Phosphate Buffer Containing D_2O

D_2O concentration in buffer, atom per cent excess deuterium (A)	Cholesterol, atom per cent excess deuterium (B)	Per cent deuterium in cholesterol derived from D_2O $\frac{B}{A} \times 100$	Fatty acids, atom per cent excess deuterium
8.3	0.58	7.0	0.028
8.4	0.72	8.6	0.055
11.0 (+20 mg. non-isotopic Na acetate)	0.55	5.0	0.053
16.4	1.80	11.0	0.180

As is the case in the intact animal, deuterio cholesterol is formed in a medium containing heavy water (Table IV). The effect has previously been ascribed to the uptake of hydrogen from the body fluids in the course of the

reduction of the carbonyl groups of acetic acid (3). In the present experiments, this process occurred in the absence of added non-isotopic acetate as well as in its presence, indicating that the slice either contains or produces during incubation sufficient quantities of acetic acid.

While synthesis could be demonstrated qualitatively in every experiment, the quantities of isotope incorporated into cholesterol varied appreciably. However, in parallel runs in which portions of pooled slices from several rat livers were incubated the level of isotope in cholesterol varied but little. The optimal conditions for cholesterol synthesis *in vitro* are still to be ascertained.

TABLE V

Atom Per Cent Excess Deuterium in Cholesterol Isolated from Liver Slices of Rats Varying in Weight

Aerobic incubation in phosphate buffer with deuterio acetate containing 59.0 atom per cent excess deuterium.

Weight of rats	Buffer volume	Atom per cent excess deuterium	
		Cholesterol	Fatty acids
gm.	*ml.*		
230	50	0.09	0.023
230	50	0.11	
230	50	0.07	0.001
230	50	0.07	
170	50	0.23	
170	50	0.28	
150	20	0.46	
130	20	0.73	0.050
120	10	1.30	0.045

The effect has not been investigated systemically, but the data (Table V) suggest a correlation between the age of the animals and the isotope concentration in cholesterol.

Synthesis of Cholesterol in Various Organs—Formation of deuterio cholesterol on incubation with isotopic acetate could be demonstrated with liver tissue only. But little isotope was introduced into cholesterol when kidney, spleen, gastrointestinal tract, or testes, organs from which slices can be readily prepared, were incubated under conditions which led to the formation of deuterio cholesterol by liver slices (Table VI). These findings do not by themselves establish the liver as the sole organ capable of synthesizing cholesterol. It can be estimated, however, that the rate at which this process occurs in liver *in vitro* is sufficiently rapid to fulfil the cholesterol requirements of the whole rat organism. Further support for the conten-

tion that the liver occupies a dominant position as a site of cholesterol synthesis can be derived from the finding that the concentrations of deuterium in cholesterol are higher in liver than in some sixteen other organs of rats receiving deuterio acetate.[4]

We have tested the effect of adenosine triphosphate (ATP) on cholesterol synthesis in liver slices. As sodium fluoride must be added in order to prevent rapid destruction of this labile compound, we also tested the effect of NaF on this reaction. In a series of experiments in which pooled slices were employed it was found that the addition of 35 mg. of NaF lowered the deuterium concentration from 0.73 to 0.13 atom per cent excess. A similar depression of isotope concentration (0.08 atom per cent excess) was observed when 40 mg. of ATP plus 35 mg. of NaF were added to the medium. No decision can be made on the basis of these results as to whether ATP is involved in this reaction, since fluoride alone inhibits the system. ATP

TABLE VI

Isotope Concentration in Cholesterol Isolated from Slices of Various Rat Organs

Incubation in phosphate buffer with deuterio acetate containing 59.0 atom per cent excess deuterium.

Organ	Atom per cent excess deuterium	
	Experiment 1	Experiment 2
Liver..........................	0.460	0.360
Gastrointestinal tract..........	0.014	0.042
Spleen.........................	0.039	0.012
Kidney.........................	0.033	0.012
Testes.........................		0.006

and NaF alone or in combination were without effect on cholesterol synthesis in preparations of disintegrated liver.

It has been pointed out that quantitative analysis does not reveal any change of the concentration of cholesterol in slices which are incubated either with or without added acetic acid. On the other hand, the present experiments with heavy water and labeled acetic acid clearly demonstrate the occurrence of sterol synthesis under these conditions. These two apparently contradictory findings may be reconciled by either one of the two following assumptions. (1) The total quantity of cholesterol synthesized represents an increase in concentration which lies within the limits of error of existing methods of analysis. The maximum isotope concentration which we have observed in the experiments with acetic acid is equivalent to the formation of 4 per cent of the cholesterol originally present, a change

[4] Unpublished experiments, K. Bloch and D. Rittenberg.

which is not detectable with certainty by quantitative analysis. On the other hand, in the experiments which were carried out with buffer containing D_2O, cholesterol contained 5 to 11 per cent of the maximum deuterium concentration, and if the latter values were actually due to an increase in the concentration of cholesterol, the change should be detectable by quantitative analysis. (2) Our data may relate to a process involving cholesterol synthesis without change in the total quantity of this substance. If an amount of cholesterol equivalent to that synthesized simultaneously undergoes chemical alteration, the constancy of the cholesterol content of the liver tissue would merely reflect the dynamic equilibrium which is known to exist in the intact animal.

The fatty acids isolated from the incubated liver slices contained considerably lower isotope concentrations than the cholesterol formed under the same conditions (Tables I, IV, and V). However, in the intact animal,[5] fatty acids are synthesized more rapidly than cholesterol, as is evident from the much higher isotope concentrations in the fatty acids of animals whose body fluids are enriched with heavy water or which receive acetic acid containing C^{13}. In the *in vitro* experiments with liver slices, conditions appear to be far more favorable for the synthesis of cholesterol than for that of fatty acids. It cannot even be decided whether the observed uptake of isotope in the fatty acids results from synthesis of the carbon chain, or whether it is due merely to hydrogenation of unsaturated acids.

SUMMARY

1. Slices of rat liver were incubated. aerobically in phosphate buffer in the presence of D_2O, deuterio acetate, or acetate containing deuterium in the methyl group and C^{13} in the carboxyl group.

2. The cholesterol isolated from slices incubated with D_2O contained deuterium, and both deuterium and C^{13} after incubation with isotopic acetate, demonstrating the *in vitro* synthesis of cholesterol.

3. No synthesis took place under anaerobic conditions, in preparations of disintegrated liver, nor in slices of kidney, testes, spleen, or gastrointestinal tract.

4. The fatty acids isolated from liver slices in which isotopic cholesterol had been formed contained small but significant concentrations of deuterium.

[5] The fact that in the intact mouse and rat deuterio acetate gives rise to higher isotope concentrations in cholesterol than in the fatty acids, although the latter are more rapidly regenerated, may be due to greater loss of carbon-bound deuterium in the process of fat synthesis than in the synthesis of cholesterol.

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BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

I. SOME TRANSFORMATIONS INVOLVING ASPARTIC ACID

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Preparation of analogues of metabolites to test their ability to inhibit growth of microorganisms has been recognized in recent years as an approach to the problem of seeking out new chemotherapeutic agents (1–3). Amino acids are among the bacterial growth factors for which antagonistic analogues have been prepared. Antagonisms between ethionine and methionine (4, 5), between α-amino-γ-methoxybutyric acid and methionine (6), between β-2-thienylalanine and phenylalanine (7), and between either β-aminobutyric acid or isoserine and β-alanine (8) have been reported.

In the present contribution, dealing with the mechanism of competitive inhibition and its utilization in elucidation of biochemical transformations in growing organisms, it is proposed that (*a*) the antibacterial index (see Table I) is a function of the inhibited enzyme system which is the limiting factor for growth of the organism; (*b*) upon supplying the product of this specific limiting enzyme system to the organism in excess of growth requirements, the analogue either becomes ineffective as a growth inhibitor or affects at higher concentrations another enzyme system, in which case a higher antibacterial index, corresponding to this second enzyme system, is obtained; (*c*) precursors of the metabolite may be effective in preventing inhibition of growth by the analogue over wide ranges of concentration.

Applying these proposals to the investigation of the competitive inhibition of aspartic acid utilization by *dl*-"*para*"-hydroxyaspartic acid has indicated that for *Escherichia coli* glutamic acid is a limiting precursor of aspartic acid (by transamination) and that the β-alanine moiety of pantothenic acid is a product of aspartic acid metabolism.

The fundamental new observations upon which the present deductions and interpretations rest are as follows: (*a*) *dl-para*-Hydroxyaspartic acid inhibits the growth of *Escherichia coli*. This inhibition is completely prevented by aspartic acid and is competitive in nature; *i.e.*, a defined inhibition occurs at a definite ratio of analogue to aspartic acid. (*b*) The competitive inhibition of growth of *Escherichia coli* by hydroxyaspartic acid is also prevented by glutamic acid over wide ranges of concentration.

(c) Pantothenic acid alters the inhibition in a characteristic manner, namely, that of changing the antibacterial index (9) to a higher value. Details of the experiments and interpretations concerning these transformations and of additional competitive inhibitions are presented below.

<div align="center">EXPERIMENTAL</div>

dl-para-Hydroxyaspartic Acid—*dl-para*-Hydroxyaspartic acid was prepared by the method of Dakin (10). Only this isomer was used in the tests described in this investigation.

meso-Diaminosuccinic Acid—The less soluble of the two optically inactive forms of diaminosuccinic acid was prepared as described by Lehrfeld (11). Work of Farchy and Tafel (12) has indicated that this isomer is *meso*-diaminosuccinic acid.

Testing Methods—Tests with *Leuconostoc mesenteroides* were carried out as described by Hac and Snell (13) for aspartic acid assay.

For tests with *Escherichia coli*, an inorganic salts-glucose medium was prepared as follows: Na_2SO_4, anhydrous, 1 gm., NH_4Cl 1 gm., K_2HPO_4 0.8 gm., $MgSO_4 \cdot 7H_2O$ 80 mg., glucose 2 gm., $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 20 mg., and casein, digested with trypsin, 200 γ were dissolved in water, diluted to 100 cc., and filtered. For assays, 5 cc. of this double strength medium were added to test materials in 5 cc. of water; the tubes were capped, autoclaved 10 minutes at 15 pounds steam pressure, and inoculated. Time and temperature of incubation are given with Tables I to VI.

The organism was a strain of *Escherichia coli* carried in this laboratory on yeast extract-glucose agar (1 per cent glucose, 1 per cent yeast extract, and 1.5 per cent agar). Although this strain would not grow initially on the inorganic salts-glucose medium, it grew very rapidly on a previously described complete medium (14). The organism was adapted to the simple medium gradually over a period of several weeks by decreasing the proportion of this complex medium and increasing the inorganic salts-glucose medium which was supplemented with 10 mg. of aspartic acid per 10 cc. The organism was then carried by daily transfers on the latter medium supplemented with 0.15 cc. of the complete medium per 10 cc. After 16 to 24 hours growth, the cells were centrifuged, washed once with 10 cc. of 0.9 per cent sodium chloride, and resuspended in 10 cc. of the saline. One drop of a 1:20 dilution of this saline suspension was used to inoculate each culture tube.

<div align="center">Results</div>

From Table I it is evident that *dl-para*-hydroxyaspartic acid possessed no growth-stimulating properties, but on the contrary was toxic for *Escherichia coli*. The inhibition of growth was prevented by added aspartic acid,

and as the amount of aspartic acid was increased, the level of hydroxy-aspartic acid necessary to inhibit growth increased. Even large amounts, more than 1 mg. per cc. of the analogue, were not inhibitory in the presence of sufficient amounts of aspartic acid. The molar ratio of hydroxyaspartic acid to aspartic acid necessary for maximum inhibition (the antibacterial index (9)) remained constant at a value of 10 to 15 over a wide range of concentrations. As judged from the amount of hydroxyaspartic acid required to inhibit growth in the absence of added aspartic acid, the maxi-

TABLE I

Growth Inhibition by Hydroxyaspartic Acid* and Its Reversal by l(+)-Aspartic Acid

Test organism, *Escherichia coli;* incubated 16 hours at 37–38°.

.(+)-Aspartic acid	Hydroxyaspartic acid*	Galvanometer reading†	l(+)-Aspartic acid	Hydroxyaspartic acid*	Galvanometer reading†
γ per 10 cc.	γ per 10 cc.		γ per 10 cc.	γ per 10 cc.	
0	0	43.3	100	0	44.0
0	10	42.5	100	100	37.5
0	30	36.5	100	300	21.0
0	50	13.0	100	1,000	10.0
0	100	4.0	100	2,000	3.5
10	0	42.5	300	0	44.0
10	30	41.0	300	300	40.0
10	50	40.0	300	1,000	20.5
10	100	13.9	300	3,000	10.0
10	200	4.0	300	5,000	2.0
30	0	43.7	1000	0	45.0
30	50	41.0	3000	0	47.7
30	100	39.0	300	10,000	2.8
30	200	13.0	1000	10,000	8.5
30	300	11.5	3000	10,000	51.6
30	500	4.5			

Antibacterial index (C_I/C_M) = 10 to 15. The antibacterial index (9) is the ratio of the concentration of inhibitor (C_I) to that of the essential metabolite (C_M) at which complete inhibition of the test organisms results.

* Less soluble racemic isomer designated as *dl-para*-hydroxyaspartic acid by Dakin (10).

† A measure of culture turbidity; distilled water reads 0, an opaque object 100.

mum concentration of aspartic acid which *Escherichia coli* produced in the cell was approximately equivalent to an outside concentration of 10 γ per 10 cc. of medium.

Table II shows that *Leuconostoc mesenteroides*, which requires added aspartic acid for growth, not only did not utilize hydroxyaspartic acid in place of aspartic acid, but the analogue inhibited competitively the response to aspartic acid. The molar ratio for maximum inhibition was 60 to 200 for this organism.

meso-Diaminosuccinic acid also inhibited growth of *Escherichia coli*, as shown in Table III.   The inhibition was similar to that obtained with

TABLE II

Growth Inhibition by Hydroxyaspartic Acid and Its Reversal by l(+)-Aspartic Acid
Test organism, *Leuconostoc mesenteroides;* incubated 72 hours at 30°.

l(+)-Aspartic acid	Hydroxyaspartic acid	Galvanometer reading
γ *per 5 cc.*	γ *per 5 cc.*	
0	0	5.0
0	50	5.5
0	3,000	4.0
50	0	51.2
50	100	50.5
50	300	43.0
50	1,000	25.0
50	3,000	10.5
50	10,000	6.0
500	0	52.9
500	1,000	50.0
500	3,000	49.0
500	10,000	26.2

Antibacterial index $(C_I/C_M) = 60$ to 200.

TABLE III

Growth Inhibition by meso-Diaminosuccinic Acid and Its Reversal by
l(+)-Aspartic Acid
Test organism, *Escherichia coli;* incubated 18 hours at 38–39°.

l(+)-Aspartic acid	Diaminosuccinic acid	Galvanometer reading
γ *per 10 cc.*	γ *per 10 cc.*	
0	0	49.8
0	300	51.0
0	1000	1.0
10	0	43.0
10	300	46.0
10	1000	37.0
10	3000	1.0
30	0	45.0
30	1000	44.0
30	3000	12.0
30	5000	4.0
100	5000	17.0
300	5000	49.0

Antibacterial index = 100 to 200.

hydroxyaspartic acid, but the inhibitor was somewhat less effective (antibacterial index, 100 to 200).

Partial prevention of hydroxyaspartic acid toxicity by pantothenic acid, β-alanine, and asparagine was obtained, as shown in Table IV. At low levels of hydroxyaspartic acid, the effectiveness of 1 γ of pantothenic acid was approximately equivalent to 10 γ of aspartic acid in preventing the toxicity, but at concentrations above 200 to 300 γ of hydroxyaspartic acid, pantothenic acid was completely ineffective. β-Alanine was at least as active as aspartic acid in preventing the inhibitory action of hydroxy-

TABLE IV

Comparative Effects of l(+)-Aspartic Acid, Pantothenic Acid, β-Alanine, and Asparagine on Prevention of Hydroxyaspartic Acid Toxicity

Test organism, *Escherichia coli;* incubated 16 hours at 38–39°.

Hydroxyaspartic acid	Galvanometer readings for each reversing agent				
	None	Pantothenic acid, 1 γ per 10 cc.	l(+)-Aspartic acid, 10 γ per 10 cc.	β-Alanine, 10 γ per 10 cc.	Asparagine, 10 γ per 10 cc.
γ per 10 cc.					
0	43.5	44.0	45.5	45.0	45.0
10	42.0	45.5	44.8	41.8	44.8
30	10.0	43.0	44.0	41.0	43.5
50	7.0	45.5	45.0	41.8	44.5
100	3.5	34.0	17.5	30.0	11.5
200	1.5	8.5	6.0	3.5	6.5
300	2.0	4.0	5.0	3.8	4.0
		100 γ per 10 cc.	100 γ per 10 cc.	100 γ per 10 cc.	100 γ per 10 cc.
0	46.0*	47.0	44.8	43.0	44.0
100	43.0*	44.0	42.0	39.5	22.0
300	7.5*	7.5	26.0	4.0	4.5
1,000	4.0*	5.0	15.8	4.5	5.0
2,000			4.5		
		3000 γ per 10 cc.	3000 γ per 10 cc.	3000 γ per 10 cc.	3000 γ per 10 cc.
0		45.8	48.5	46.0	48.0
10,000		5.0	47.5	5.2	4.0

* Supplemented with 10 γ of pantothenic acid per tube.

aspartic acid below levels of 200 to 300 γ per 10 cc. of analogue but was completely ineffective at higher concentrations of the inhibitor. Asparagine gave similar results but did not appear to be as active as β-alanine.

The effect of added pantothenic acid on the molar ratio of hydroxy-aspartic acid to aspartic acid for maximum inhibition is shown in Table V. Addition of 5 γ of pantothenic acid per 10 cc. resulted in a change of the antibacterial index from 3 to 20 in this and similar experiments. The anti-

bacterial index determined both with and without added pantothenic acid varied with the temperature of incubation. For example, in tests with incubation for 16 hours at 37–38° a change of antibacterial index from 10 to 30 was obtained on the addition of 5 γ of pantothenic acid per 10 cc. of medium.

Addition of 50 γ of pyridoxamine, 0.1 γ of biotin, 5 γ of riboflavin, 5 γ of nicotinic acid, 0.2 γ of folic acid, 5 γ of thiamine, or 1 γ of p-aminobenzoic

TABLE V

Effect of Pantothenic Acid on Hydroxyaspartic Acid Toxicity

Test organism, *Escherichia coli;* incubated 18 hours at 38–39°.

Hydroxyaspartic acid	l(+)-Aspartic acid	Galvanometer reading	
		Without pantothenic acid	With pantothenic acid, 5 γ per 10 cc.
γ per 10 cc.	*γ per 10 cc.*		
0	0	57.5	58.5
10	0	30.0	54.5
30	0	4.0	28.0
50	0		10.0
100	0		4.5
0	30	50.0	52.0
30	30	13.0	53.0
100	30	6.0	51.0
300	30	4.0	13.5
500	30		9.0
1000	30		4.0
0	100	53.5	52.0
100	100	19.0	53.0
300	100	4.0	39.0
1000	100		15.0
2000	100		6.0

Antibacterial index   3 without pantothenic acid, 20 with added pantothenic acid.

acid per 10 cc. of medium in place of pantothenic acid did not affect the toxicity of hydroxyaspartic acid for *Escherichia coli*.

Even though the rate of growth of the organism in a medium supplemented with all the vitamins (except pantothenic acid) in the quantities listed above was found to be greater than in a medium supplemented with pantothenic acid alone, the antibacterial index determined in the medium containing 5 γ of pantothenic acid per 10 cc. was found to be 3 to 9 times that determined in the medium supplemented with the nutrilite mixture. The latter antibacterial index was almost identical with that obtained in a medium containing none of these vitamins.

The relative ability of aspartic acid and glutamic acid to reverse the toxicity of hydroxyaspartic acid is shown in Table VI. Glutamic acid and aspartic acid were equally effective except at high concentrations, at which glutamic acid began to show decreasing activity. Oxalacetic acid, malic acid, succinic acid, and fumaric acid were found in similar tests to be completely ineffective in reversing the inhibition.

TABLE VI

Reversal of Hydroxyaspartic Acid Toxicity by l(+)-Glutamic Acid

Test organism, *Escherichia coli;* incubated 16 hours at 37°.

Hydroxyaspartic acid	Galvanometer readings for reversing agent	
	l(+)-Aspartic acid, 0 γ per 10 cc.	*l*(+)-Glutamic acid
γ *per 10 cc.*		
0	48.0	
30	48.5	
100	8.0	
300	1.0	
	30 γ per 10 cc.	30 γ per 10 cc.
0	47.5	47.3
100	40.0	43.0
300	6.0	8.0
1000	1.0	5.0
	100 γ per 10 cc.	100 γ per 10 cc.
0	48.3	47.5
100	44.5	44.6
300	21.0	27.3
1000	1.0	1.0
	300 γ per 10 cc.	300 γ per 10 cc.
0	47.0	53.1
100	46.0	48.4
300	44.0	37.0
1000	20.5	6.0
3000	1.0	1.0

Isoserine, which bears the same structural relationship to hydroxy-aspartic acid as β-alanine to aspartic acid, did not inhibit growth of *Escherichia coli* at a concentration of 10 mg. per 10 cc.

DISCUSSION

In a number of instances the amount of analogue required to cause growth inhibition is in a constant molar ratio to the amount of metabolite

present over a wide range of concentration. This is true only when the other components of the medium are the same and when the size of inoculum and time and temperature of incubation are not allowed to vary. This may be interpreted mathematically by assuming that inhibition of growth of a microorganism results from competition of the analogue with a metabolite for a specific enzyme and that the analogue-enzyme complex is not capable of carrying out the normal function of the metabolite.

Competition of an inhibitor (analogue, in our case hydroxyaspartic acid), I, with a substrate (metabolite, in our case aspartic acid), S, for an enzyme has often been represented by the following equations where P represents the product and ES and EI represent the enzyme-substrate complex and enzyme-inhibitor complex, respectively.

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$E + I \rightleftharpoons EI$$

By mass law,

$$\frac{[E][S]}{[ES]} = K_S \tag{1}$$

where K_S is the dissociation constant of the enzyme-substrate complex, and

$$\frac{[E][I]}{[EI]} \quad K_I \tag{2}$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. By dividing Equation 2 by Equation 1, one obtains

$$\frac{[I]}{[S]} = \frac{K_I[EI]}{K_S[ES]} \tag{3}$$

If $[E_t]$ represents the total enzyme concentration, both free and combined, by definition

$$[E_t] = [E] + [EI] + [ES] \tag{4}$$

In application of these equations to inhibition of growth of microorganisms, the following limitations simplify the problem. (1) Under the limiting conditions of defined basal medium, size of inoculum, and time and temperature of incubation, the total amount of growth of a microorganism is a function of the amount of product (P) formed by the inhibited enzyme reaction when this reaction becomes the limiting factor of growth, specifically when growth of the organism is being inhibited by the analogue. Since the total amount of growth is constant for a defined inhibition, the

amount of product (P) formed during the constant time of incubation is constant. Since the rate of formation of the product is directly proportional to [ES] and the time of incubation is constant, then [ES] = C_{ES}, a constant concentration of enzyme-substrate complex. (2) For concentrations of substrate and inhibitor approaching that of enzyme saturation, [E] → 0 and is negligible in comparison with [EI]. Since [ES] is constant and [E] is negligible in comparison with [EI], by assuming [E_t] to be constant in the bacterial cell during growth, it is then apparent from Equation 4 that [EI] = C_I, a constant concentration of enzyme-inhibitor complex. The assumption that [E_t] is constant in the cell during bacterial growth has been made by Wyss (15) in applying similar equations to the inhibition of growth of *Escherichia coli* by sulfanilamide to show that the inhibition was competitive. Effects of substrates on synthesis and stability of enzymes have been noted (16), but, by growing the organism in a medium containing the substrate before testing with an inhibitor, these effects presumably become negligible.

Hence, by substitution of C_{EI} and C_{ES} respectively in Equation 3, we obtain Equation 5 which may be applied to the instances under discussion

$$\frac{[I]}{[S]} = \frac{K_I C_{EI}}{K_S C_{ES}} = K \tag{5}$$

where K is the molar ratio of analogue to metabolite within the bacterial cell for a given inhibition. If it is assumed that this concentration ratio within the cell is a function of the concentration ratio in the medium, then the latter ratio is also constant for a given inhibition.

If the metabolite, S, is utilized by an organism in several enzyme systems to synthesize several products, P_1, P_2, P_3, etc., then a specific analogue, I, may be capable of preventing the conversion of S to one or more of these products. If one of these conversions, for example $S \rightarrow P_1$, is inhibited to the largest extent and becomes the limiting factor for growth, Equation 5 applies to that particular enzyme system, and the K value (becoming K_1 for this particular case) is the molar ratio for a defined inhibition. For maximum inhibition, K_1, a property of the enzyme effecting the conversion, $S \rightarrow P_1$, would indeed be the antibacterial index.

However, if P_1 is supplied in excess of growth requirements to the organism, the effect of I on the conversion, $S \rightarrow P_1$, is of no consequence in inhibiting the growth of the bacteria. If the analogue cannot combine with other enzymes which convert S to P_2, P_3, etc., the analogue becomes ineffective as a growth inhibitor. But in many cases, the analogue may also prevent other reactions, say $S \rightarrow P_3$, in which case addition of P_1 in excess of growth requirements would not render the analogue ineffective as a growth inhibitor, but on the contrary, this second enzyme system,

which becomes the limiting process for growth, is affected. Equation 5 applied to this particular enzyme system would give a higher K value, K_3, which would then be the molar ratio for a given inhibition (or in case of maximum inhibition, the antibacterial index) of the organism when grown in the presence of P_1. Thus, addition of the product (or its equivalent) of an inhibited enzyme system merely necessitates a change in ratio of analogue to metabolite sufficient to inhibit the enzyme system with the next higher K value in order again to prevent growth of the organism.

The change in molar ratio for maximum inhibition, the antibacterial index, resulting from addition of pantothenic acid (or β-alanine) to the medium is an example of the effect of addition of the product (or its equivalent) of the inhibited enzyme system to the medium. The inhibited enzyme system is no longer indispensable, since the product is already present; however, another enzyme system is affected, as is revealed by a higher antibacterial index corresponding to this other enzyme system.

Thus, aspartic acid, aside from its share in the synthesis of proteins, has been found to be an intermediate in the biological synthesis by *Escherichia coli* of essential metabolites. The functioning of the enzyme system which synthesizes the β-alanine portion of pantothenic acid is blocked by hydroxyaspartic acid reacting in competition with aspartic acid. Since isoserine, which might have been formed by decarboxylation of hydroxyaspartic acid, was ineffective in inhibiting growth, competition between isoserine and β-alanine is eliminated as a possibility; so, the decarboxylation of aspartic acid to β-alanine is the probable step in the synthesis of pantothenic acid which is blocked. These results do not preclude the possibility that the aspartic acid combines with other groupings such as the pantoyl group before decarboxylation. Asparagine is as effective as aspartic acid in preventing the toxicity of hydroxyaspartic acid at low concentrations but is not converted to any appreciable extent into aspartic acid above a definite level of the inhibitor.

Of growth factors and amino acids studied other than aspartic acid, only glutamic acid effectively prevented the toxicity of hydroxyaspartic acid over a range of concentrations. Glutamic acid was as effective as aspartic acid up to 100 γ per 10 cc. of medium. Beyond that the effectiveness of glutamic acid decreased and was not enhanced by oxalacetic acid. The most logical explanation of this effect is that the transamination reaction converting glutamic acid and oxalacetic acid to aspartic acid and ketoglutaric acid is dependent upon the amount of glutamic acid available and not upon oxalacetic acid which *Escherichia coli* presumably produces in sufficient quantities. The enzyme system may not be capable of converting large amounts of glutamic acid to aspartic acid, or large amounts of hydroxyaspartic acid may inhibit the functioning of the enzyme.

Lichstein and Cohen (17) have recently shown that *Escherichia coli*, among other organisms, readily converts glutamic acid to aspartic acid by the transamination reaction.

Although resting *Escherichia coli* cells decompose aspartic acid to fumaric acid and ammonia by a reversible process (18), fumaric acid showed no ability to reverse hydroxyaspartic acid toxicity on rapidly growing *Escherichia coli*. Succinic acid, malic acid, and oxalacetic acid were also ineffective.

This method of investigation has shown promising results in connection with the study of other transformations, details of which will be presented in forthcoming papers.

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SUMMARY

dl-para-Hydroxyaspartic acid and *meso*-diaminosuccinic acid have been found to inhibit competitively some of the functions of aspartic acid in *Escherichia coli*. Hydroxyaspartic acid also inhibited growth of *Leuconostoc mesenteroides* which requires aspartic acid for growth.

The inhibition of growth of *Escherichia coli* by hydroxyaspartic acid was also prevented over wide ranges of concentration by glutamic acid which must therefore serve as a precursor of aspartic acid. Pantothenic acid, β-alanine, and asparagine were effective in preventing the toxicity of hydroxyaspartic acid for *E. coli* only at low levels of hydroxyaspartic acid concentration. The effect of supplying pantothenic acid in the medium raised the antibacterial index to a higher value. A mathematical interpretation of the antibacterial index is given, and the following conclusions are drawn. (1) Aspartic acid is a precursor in the biosynthesis by *E. coli* of the β-alanine portion of pantothenic acid. (2) β-Alanine is also used by the organism for pantothenic acid synthesis. (3) Under the conditions of testing, asparagine is not converted to aspartic acid to any appreciable extent when the concentration of hydroxyaspartic acid is above a given level. (4) At least one additional biosynthetic transformation involving aspartic acid is prevented by sufficient amounts of hydroxyaspartic acid.

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BIOCHEMICAL TRANSFORMATIONS AS DETERMINED BY COMPETITIVE ANALOGUE-METABOLITE GROWTH INHIBITIONS

II. SOME TRANSFORMATIONS INVOLVING p-AMINOBENZOIC ACID

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The effectiveness of p-aminobenzoic acid in preventing the antibacterial action of sulfanilamide was the first basis for classifying p-aminobenzoic acid as an essential metabolite (1). However, substances other than p-aminobenzoic acid have been reported to "antagonize" the inhibitory action of sulfanilamides (2–4).

Using *Escherichia coli* as a test organism, Kohn and Harris (5) studied the "antagonism" of sulfanilamide action by methionine and postulated a metabolic interrelationship between p-aminobenzoic acid and methionine. In reporting "antagonistic" action of purine bases toward sulfanilamide inhibition of lactic acid bacteria, Snell and Mitchell (6), referring to the work of Kohn and Harris (5), proposed that this interrelationship could be extended to include the purine bases, adenine, guanine, xanthine, and hypoxanthine.

Regarding the mechanism of competitive inhibition of growth by analogues of metabolites and its utilization in studying biochemical transformations, it was proposed in Paper I (7) that the antibacterial index is determined by the inhibited enzyme system which is the limiting factor for growth of the organism. Upon supplying the product of this specific limiting enzyme system to the organism in excess of growth requirements, the analogue either becomes ineffective as a growth inhibitor or at higher concentrations affects another enzyme system to which a new antibacterial index corresponds.

In the present investigation, the competitive inhibitions of p-aminobenzoic acid metabolism by sulfanilamide and by 4-amino-2-chlorobenzoic acid (8) and so called "antagonists" of their inhibitory action have been studied with respect to the above proposals.

For *Escherichia coli*, it has been found that the antibacterial index of 4-amino-2-chlorobenzoic acid is related to the enzyme system which synthesizes methionine. If methionine was supplied in the medium, the analogue became ineffective as a growth inhibitor. Also it was found that the antibacterial index for sulfanilamide inhibition of *Lactobacillus arab-*

inosus 17-5 was changed to a higher value by supplying purine bases in the medium.   The lowest antibacterial index obtained in inhibiting growth of *Escherichia coli* with sulfanilamide corresponded to the enzyme system which synthesizes methionine.   The next higher antibacterial index obtained in a medium containing methionine was associated with the enzyme system synthesizing the purine bases, guanine or xanthine, or products into which the purines could be converted by the organism.   In a medium containing both purine bases and methionine, a still higher antibacterial index was obtained which corresponds to a third unexplored enzyme system in which sulfanilamide also competitively inhibited the functioning of *p*-aminobenzoic acid.   Details of these experiments and their significance

TABLE I

Effect of Increased Amounts of Adenine Sulfate on Sulfanilamide Toxicity for Lactobacillus arabinosus 17-5

Incubated 16 hours at 31–32°.

Adenine sulfate	*p*-Amino-benzoic acid	Sulfanilamide per 10 cc.						
		0 γ	3 γ	10 γ	30 γ	100 γ	300 γ	1000 γ
		Galvanometer reading*						
γ per 10 cc.	*γ per 10 cc.*							
0	0.1	81	73	. 8	2	2		
30	0.1	87	81	74	19	2		
100	0.1 ·	90	90	87	55	3		
300	0.1	89	88	75	11	2		
1000	0.1	89	85	51	30	2		
. 0	1.0	80			38	5	2	2
30	1.0	86			83	78	40	2
100	1.0	90			88	88	79	8
300	1.0	90			90	87	72	4
1000	1.0	90	..		90	88	65	2

* A measure of culture turbidity; distilled water reads 0, an opaque object 100.

in the study of biochemical transformations and in the search for chemotherapeutic agents are presented below.

EXPERIMENTAL

Testing Methods—Inocula, conditions of testing, and basal medium for *Lactobacillus arabinosus* 17-5 were essentially those described by Snell, Guirard, and Williams (9) but modified as indicated by Snell and Mitchell (6).   Tests with *Escherichia coli* were carried out as previously described (7).   Time and temperature of incubation are given with Tables I to V.

Results

The effect of increased amounts of adenine sulfate on sulfanilamide toxicity for *Lactobacillus arabinosus* is shown in Table I.   The amount of

sulfanilamide required for maximum inhibition in the presence of adenine sulfate was 10 times that required for maximum inhibition in the absence of this purine.   The effectiveness of adenine sulfate did not appreciably with increases in concentration above the initial 30 γ per 10 cc. even to concentrations of 1 mg. per 10 cc.

TABLE II

Effect of Purine Bases on Sulfanilamide Toxicity for Lactobacillus arabinosus 17-5
Incubated 16 hours at 31–32°.

Sulfanilamide	*p*-Aminobenzoic acid	Galvanometer reading		
			With added purine bases	
		Without added purine bases	Adenine sulfate, guanine hydrochloride, xanthine, 10 γ each per 10 cc.	Adenine sulfate, 100 γ per 10 cc.
γ *per 10 cc.*	γ *per 10 cc.*			
0	0.01	76	82	83
0.3	0.01	48	80	72
1.0	0.01	8	69	73
3	0.01	2	31	17
10	0.01	2	5	5
0	0.1	81	86	90
3	0.1	73	85	90
10	0.1	8	78	87
30	0.1	2	33	55
100	0.1	2	3	3
0	1.0	80	84	90
30	1.0	38	82	88
100	1.0	5	72	88
300	1.0	2	20	79
1000	1.0	2	2	8
0	3.0	85	85	89
100	3.0	63	85	89
300	3.0	4	78	88
1000	3.0	2	46	70
3000	3.0	2	4	29
Antibacterial index		100	1000	1000

The effect of purine bases on sulfanilamide toxicity for *Lactobacillus arabinosus* as shown in Table II is that of changing the molar ratio for maximum inhibition of growth, the antibacterial index, from 100 to 1000. A mixture of purine bases, adenine sulfate, guanine hydrochloride, and xanthine was no more effective than adenine sulfate alone.   The antibacterial indices were determined over a 300-fold increase in *p*-aminobenzoic acid concentration.   Methionine in independent experiments

involving a medium containing hydrolyzed casein had no effect on sulfanila-
mide toxicity for *Lactobacillus arabinosus* either in the presence or absence
of purine bases.

As shown in Table III, the molar ratio for maximum inhibition of growth
of *Escherichia coli* with sulfanilamide was 3000 determined in the absence
of methionine and 10,000 in the presence of methionine.

TABLE III

Effect of Methionine on Sulfanilamide Toxicity for Escherichia coli

Incubated 16 hours at 37–38°.

Sulfanilamide	p-Aminobenzoic acid	Galvanometer reading	
		Without added dl-methionine	With added dl-methionine, 100 γ per 10 cc.
γ per 10 cc.	γ per 10 cc.		
0	0	50	52
100	0	48	50
300	0	8	49
1,000	0	2	25
3,000	0	1	4
0	0.3	49	51
300	0.3	45	49
1,000	0.3	4	46
3,000	· 0.3	2	10
10,000	0.3	2	3
0	1.0	49	50
1,000	1.0	44	48
3,000	1.0	5	43
10,000	1.0	2	5
30,000	1.0	1	1
0	3.0	47	50
3,000	3.0	41	48
10,000	3.0	6	41
30,000	3.0	2	7
50,000	3.0	1	1
Antibacterial index		3000	10,000

· The effect of purine bases in the presence and absence of methionine on
sulfanilamide toxicity for *Escherichia coli* is shown in Table IV.   In the
absence of methionine, purine bases had no effect on the toxicity of the
sulfanilamide, but in the presence of methionine, the addition of purines
changed the antibacterial index from 3000 to 30,000.   Xanthine and gua-
nine are apparently interconvertible by *Escherichia coli*, as each alone was
as effective as a mixture of both.   In other tests, adenine not only did not
"antagonize" sulfanilamide action but slightly increased the toxic effects.

The antibacterial index varies with size of inoculum and time and temperature of incubation, and variations such as 3000 to 10,000 in separate tests are not uncommon unless an unusual effort is made to keep the con-

TABLE IV

Effect of Purine Bases in Presence and Absence of dl-Methionine on Sulfanilamide Toxicity for Escherichia coli

Incubated 16 hours at 38–39°.

			Galvanometer reading			
				Purine bases added		
Sulfanilamide	p-Amino-benzoic acid	dl-Methionine	No purine bases added	Xanthine, 100 γ per 10 cc.	Guanine hydrochloride, 100 γ per 10 cc.	Xanthine and guanine hydrochloride, 100 γ each per 10 cc.
γ per 10 cc.	γ per 10 cc.	γ per 10 cc.				
0	0	0	46	48	46	54
100	0	0	44	47	47	49
300	0	0	3	5	4	2
0	0	100	48	51	48	50
300	0	100	45	48	47	48
1,000	0	100	3	15	23	22
3,000	0	100	2	8	10	10
10,000	0	100	2	3	3	6
0	0.3	100	48	52	49	50
300	0.3	100	49	49	48	48
1,000	0.3	100	3	47	44	43
3,000	0.3	100	2	35	25	23
10,000	0.3	100	2	8	9	9
30,000	0.3	100	2	5	4	5
0	1.0	100	47	50	48	51
1,000	1.0	100	47	47	44	48
3,000	1.0	100	3	44	45	45
10,000	1.0	100	2	13	14	19
30,000	1.0	100	2	6	7	9
50,000	1.0	100	2	3	4	4
0	3.0	100	48	50	48	50
3,000	3.0	100	49	50	47	48
10,000	3.0	100	3	45	43	45
30,000	3.0	100	3	12	14	13
50,000	3.0	100	3	4	3	6
·ial index..............			3000	30,000	30,000	30,000

ditions exactly the same; hence, all data for comparative purposes must be obtained in a single test. This type of variation accounts for the difference in antibacterial index obtained with *Escherichia coli* in the presence of methionine in separate tests, Tables III and IV.

The molar ratio of 4-amino-2-chlorobenzoic acid to *p*-aminobenzoic acid for maximum inhibition of *Escherichia coli* is shown in Table V to be about 3000. When methionine was added to the growth medium, no effective inhibition of growth was obtained even when the inhibitor was at a concentration of 10 mg. per 10 cc. of medium. In a different experiment, 4-amino-2-chlorobenzoic acid did not inhibit growth of *Lactobacillus arabinosus* at a concentration of 10 mg. per 10 cc. in the presence of 0.1 γ of *p*-aminobenzoic acid per 10 cc. of medium.

<div align="center">TABLE V</div>

Effect of Methionine on Toxicity of 4-Amino-2-chlorobenzoic Acid for Escherichia coli

Incubated 16 hours at 38–39°.

4-Amino-2-chlorobenzoic acid	*p*-Aminobenzoic acid	Galvanometer reading	
		Without added *dl*-methionine	With added *dl*-methionine, 100 γ per 10 cc.
γ *per 10 cc.*	γ *per 10 cc.*		
0	0	48	50
100	0	45	49
300	0	9	48
1,000	0	1	48
3,000	0		45
10,000	0		41
0	0.3	49	
300	0.3	44	
1,000	0.3	·1	
0	1.0	48	
1,000	1.0	44	
3,000	1.0	1	
0	3.0	48	
3,000	3.0	43	
10,000	3.0	1	
Antibacterial index		3000	>100,000

<div align="center">DISCUSSION</div>

In clarifying the discussion of some so called "antagonists" of competitive analogue-metabolite growth inhibitions, it is desirable to introduce a new term, product inhibition index. The term is defined as the molar ratio of analogue to metabolite at which the rate of synthesis of the product is the limiting process for growth and is reduced to such an extent as to prevent growth of the organism in a medium free of the product.

To illustrate its use, we may say that the methionine inhibition index of

Escherichia coli in the case of sulfanilamide·is the molar ratio of sulfanilamide to *p*-aminobenzoic acid (in a methionine-free medium) which inhibits the synthesis of methionine sufficiently to stop growth of the organism.

For a given analogue of a metabolite in a medium free of products derived from the metabolite, the lowest product inhibition index is the antibacterial index. If some of the products are supplied in excess of growth requirements, the lowest inhibition index of the products which are not supplied becomes the antibacterial index. If all the products are supplied in excess, the analogue becomes ineffective as a growth inhibitor.

The product inhibition index varies just as the antibacterial index with size of inoculum and time and temperature of incubation; so slight variations among separate tests are noted unless special precautions are taken to keep these constant.

The purine base inhibition index of *Lactobacillus arabinosus* was 100 under the conditions of testing shown in Table II. When adenine or a mixture of purine bases was supplied, another product inhibition index, which amounted to 1000, became the antibacterial index. According to the work of Snell and Mitchell (6), *Lactobacillus arabinosus* appears to be capable of interconverting xanthine, hypoxanthine, adenine, and guanine. Thus, these purine bases in general are products of metabolism involving an enzyme system in which *p*-aminobenzoic acid functions.

In our experiments, the methionine inhibition index of *Escherichia coli* for sulfanilamide was about one-third the purine base inhibition index which was in turn one-tenth as large as another inhibition index of a product as yet unknown. Hence, the antibacterial index varied 30-fold, depending upon whether or not these two products were present in the growth medium. For *Escherichia coli*, guanine and xanthine appear to be interconvertible, but adenine was found to enhance slightly sulfanilamide toxicity for the organism. Hence, methionine and the purine bases, guanine and xanthine, are products of enzyme systems in which *p*-aminobenzoic acid functions.

Kohn and Harris (5) earlier concluded that *p*-aminobenzoic acid functions in the synthesis of methionine. The experimental data upon which their conclusion was based are as follows: (*a*) Methionine prevented sulfanilamide toxicity for *Escherichia coli* only below a definite level of inhibitor. (*b*) Inhibitory action of ethionine was prevented by methionine, but not by *p*-aminobenzoic acid. The possibility that methionine and another material could have been precursors for *p*-aminobenzoic acid was discarded, because *p*-aminobenzoic acid did not antagonize the toxicity of ethionine. In the light of our findings, the basis for their conclusion does not appear completely valid. For example, purine bases and methio-

nine are products of enzyme systems in which p-aminobenzoic acid functions in *Escherichia coli*, but purine bases, a product or its equivalent, do not exert an "antisulfanilamide" effect in the absence of methionine. Hence, the failure of p-aminobenzoic acid to prevent ethionine toxicity did not eliminate the possibility of p-aminobenzoic acid being a product of methionine metabolism.

For 4-amino-2-chlorobenzoic acid, the methionine inhibition index of *Escherichia coli* was 3000, as shown in Table V. If methionine was supplied in excess of growth requirements, the analogue was ineffective as a growth inhibitor; hence 4-amino-2-chlorobenzoic acid is a specific inhibitor of the functioning of p-aminobenzoic acid in methionine synthesis.

An analogue of a metabolite may not react with all the enzymes with which the metabolite reacts, and the enzyme-analogue complexes which are formed may or may not function as the enzyme-metabolite complexes. Hence, analogues may be more specific than the metabolite itself; so in the study of growth inhibitions by analogues of metabolites, knowledge of the composition of the growth media is imperative. For example, if the inhibiting properties of 4-amino-2-chlorobenzoic acid had been studied in a medium containing methionine in excess of growth requirements, it would have been found to be completely ineffective as a growth inhibitor. By improper choice of media, including those of unknown composition, powerful inhibitors of synthesis of some of the products included in the media may be overlooked, because under these conditions inhibitors may become completely ineffective. If the effective medium at the site of an infection lacks constituents which are present in the medium used for *in vitro* tests, valuable chemotherapeutic agents may be completely overlooked. Conversely, chemotherapeutic agents which are worthless *in vivo* may appear highly potent *in vitro* if they are tested in media lacking products which are present at the site of an infection. Ideally, therefore, *in vitro* tests for chemotherapeutic agents should be made in a medium resembling as closely as possible that present at the site of infection. The lack of correspondence between *in vitro* and *in vivo* tests is probably due to a large extent to a failure to observe this principle.

SUMMARY

Some "antagonists" of sulfanilamide action have been found to be products or their equivalent of enzymatic reactions in which p-aminobenzoic acid functions.

The term, product inhibition index, is proposed and defined as the molar ratio of analogue to metabolite at which the rate of synthesis of the product is the limiting process for growth and is reduced to such an extent as to prevent growth of the organism in a medium free of the product.

For sulfanilamide, the methionine inhibition index of *Escherichia coli* is one-third of the purine base inhibition index, and the purine base inhibition index is one-tenth that of the inhibition index of another product as yet unknown. Thus, p-aminobenzoic acid functions in the synthesis of methionine and purine bases, xanthine and guanine, in *E. coli*. 4-Amino-2-chlorobenzoic acid is shown to be a specific inhibitor of methionine synthesis by *E. coli*. The purine base inhibition index of *Lactobacillus arabinosus* is one-tenth that of the inhibition index of another unknown product. Hypoxanthine, xanthine, guanine, and adenine, all being interconvertible, are products of an enzyme system of *Lactobacillus arabinosus* in which p-aminobenzoic acid functions.

It is indicated that knowledge of the composition of media is imperative in seeking out competitive inhibitors of metabolite functioning. Valuable chemotherapeutic agents may be completely overlooked if the medium used for *in vitro* tests contains products not present in the effective medium at the site of the infection to be treated. Conversely, chemotherapeutic agents which are worthless *in vivo* may appear highly potent *in vitro* if they are tested in media lacking products which are present at the site of an infection.

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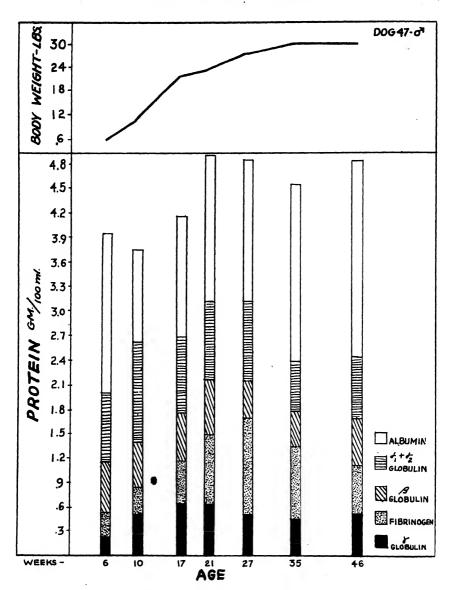
FIG. 1. Changes in the plasma protein fractions of Dog 47 during growth. Observations by the electrophoresis method in phosphate buffer of pH 7.8, ionic strength 0.16.

the body weight and the plasma protein level. In three of the four dogs there was a decrease in the total plasma protein during the 1st month following weaning and in all cases there was a fall in the albumin level.

The albumin decrement was 0.7, 0.1, 0.3, and 0.5 gm. per 100 ml., respectively. The γ-globulin increased to 2.5 times the initial value during the same period. The other globulins showed no consistent change.

When the animals were between 3 and 5 months of age there was a large increment in both the total protein and the albumin. The average increment in total protein was 0.7 gm. per 100 ml., and in albumin 0.45 gm. per 100 ml. After the 5th month, when the period of rapid growth was over, there was a lag in the rate of total protein and albumin increase.

The fibrinogen showed a definite upward trend in all cases until the 5th month. Between the 5th and 6th months, a particularly large increment was observed. By the 10th month, the level had decreased to 0.90, 0.78, and 0.65 gm. per 100 ml. in three of the dogs. The fourth dog had the mange during this period and showed unusually high fibrinogen values of 1.7 gm. per 100 ml.

Two of the dogs developed mange when they were 7 months old. This infection may possibly have modified the level of the protein fractions. One recovered within a few weeks. The other had a skin eruption for the remainder of the period of observation.

In each case there was a slight decrement (0.35, 0.23, 0.23, 0.36 gm. per 100 ml. respectively) in the total protein between the 6th and the 9th month, but there was at the same time a definite increase in albumin (0.43, 0.23, 0.34, 0.41 gm. per 100 ml. respectively).

When the animals were fully grown the total plasma protein had increased between 1.0 and 1.5 gm. per 100 ml. from the level at weaning. Of this 0.63, 0.50, 0.48, and 0.31 gm. per 100 ml. respectively were albumin. In no case did the β-globulin or the α_1 plus α_2-globulins show significant changes from the initial level. There was, however, a definite increase in the γ-globulin level. The average increase from the level at weaning was 110 per cent.

DISCUSSION

Swanson and Smith (2) found that in rats the most pronounced increment in plasma protein nitrogen occurred in early life, from weaning until the rats were 50 days old. After that the increase was very gradual and the data indicated that an approximately uniform level was maintained in adult life. Our findings on dogs are similar, except for an initial slight drop in the total protein, and definite decrease in the albumin immediately following weaning.

The changes observed between the 6th and the 8th month probably correspond with those observed in rats at 60 to 80 days by Hatai and at 100 days by Swanson and Smith, and probably parallel the onset of sexual maturity.

SUMMARY

The total protein and Tiselius electrophoretic fractions were determined on the plasma of four dogs from the time the animals were weaned until they were fully grown. The total protein showed an initial slight drop, then a rapid increase during the period of most rapid growth. The γ-globulin increased markedly during this time. The albumin decreased during the 1st month, and returned to the initial level at about the 6th month. Between the 6th and 8th months a large increase in the albumin was observed.

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A STUDY OF THE REQUIREMENTS OF WHITE LEGHORN CHICKS FOR NEW AND UNIDENTIFIED MEMBERS OF THE VITAMIN B COMPLEX

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Reports published during the last few years indicate that there have been isolated from natural sources at least three compounds, all included under the term "folic acid," which have growth and antianemia activity for chicks. These compounds are vitamin B_c[1] and vitamin B_c conjugate, both isolated by a group of workers in the Parke, Davis laboratories (2–4), and *Lactobacillus casei* ε factor from fermentation products (LCF) first obtained by workers in the Lederle Laboratories (12). "Folic acid," as originally described by Mitchell, Snell, and Williams (5), seems now to be accepted as an impure preparation of vitamin B_c. Other factors in this group of compounds, such as the *Streptococcus lactis* R factor (SLR) of Keresztesy *et al.* (6), factors R and S of Schumacher, Heuser, and Norris (7), and vitamins B_{10} and B_{11} of the Wisconsin workers (8) either have not been obtained in pure form, or adequate reports have not been made which would definitely indicate their rôle in chick nutrition.

In the case of vitamin B_c only reports on its activity in preventing anemia in chicks have been extensive (2, 9–11, 1). Its effect on growth and feathering is not clear from published results because the growth obtained in most instances with pure preparations fell far short of that which might be expected from the use of a good practical diet. From the reports of Binkley *et al.* (3) and Pfiffner and coworkers (4) it may be accepted that the chick activity of vitamin B_c conjugate is identical in all respects with that of vitamin B_c. Little or no data on LCF have been published by Hutchings *et al.* (12), the discoverers of this compound, but recent reports of Scott and associates (13, 14) indicate that LCF requires the presence of pyridoxic acids in order to show significant chick activity.

We have carried out numerous chick experiments on the value of certain natural materials, of concentrates made from some of these, and of LCF and pyridoxic acids as supplements to a highly purified basal diet similar in all respects to that used by Briggs *et al.* (15). Some data are presented here in the hope that they may contribute substantially to a better understanding of the value of some of these new and unidentified B complex vitamins in chick nutrition.

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[1] Vitamin B_c has apparently been synthesized by Augier *et al.* (1), although the synthetic product is called "liver *L. casei* factor" by the authors.

All experiments were conducted with day-old single comb white Leghorn cockerels from a single hatchery and a single flock. The basal diet which is similar to that used by the Wisconsin workers (15) is listed in Table I.

Each bird received semiweekly 1 drop of fortified halibut liver oil which contained in each gm. 5000 A. O. A. C. units of vitamin D, 60,000 U. S. P. units of vitamin A, and 15 mg. of added *dl-α*-tocopherol acetate.

TABLE 1

Basal Diet

	per cent
Gelatinized starch (Corn Products Refining Company).	52.70*
Casein (Smaco vitamin test)	18.00
Gelatin (The Wilson Laboratories)	10.00
Soy bean oil (crude, expeller grade)	5.00
l(−)-Cystine	0.30
KI supplement†	1.00
Salts supplement‡	10.00
$CaHPO_4 \cdot 2H_2O$	1.00
Biotin supplement§	1.00
Vitamin " ‖	1.00

* Total carbohydrate adjusted to 60.42 per cent, account being taken of starch in the supplements.

† 0.400 gm. of KI made up to 100 gm. with starch and mixed by grinding in a pebble mill.

‡ 8.364 gm. of NaCl, 16.112 gm. of K_2HPO_4, 3.748 gm. of $CaHPO_4 \cdot 2H_2O$, 5.096 gm. of $MgSO_4 \cdot 7H_2O$, 14.988 gm. of $CaCO_3$, 1.376 gm. of $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.400 gm. of $MnSO_4 \cdot 4H_2O$, 0.014 gm. of $ZnCl_2$, and 0.016 gm. of $CuSO_4 \cdot 5H_2O$, made up to 100 gm. with starch and mixed by grinding in a pebble mill.

§ 1.50 mg. of Merck's crystalline biotin dissolved in water, sprayed on starch, dried, made up to 100 gm. with starch, and mixed by grinding in a mill.

‖ 30.0 mg. of thiamine hydrochloride, 60 mg. of riboflavin, 40 mg. of pyridoxine hydrochloride, 300 mg. of *dl*-calcium pantothenate, 1.00 gm. of nicotinic acid, 50 mg. of 2-methyl-1,4-naphthoquinone, 15 gm. of choline chloride, and 10 gm. of *i*-inositol, made up to 100 gm. with gelatinized starch and mixed by grinding in a mortar.

When supplements of unidentified factors or crystalline compounds other than those listed above were added to the diet, they were mixed with starch and the completed supplement was added at the expense of the gelatinized starch.

Both yeast extract[2] and liver fraction L[3] have been used as our control

[2] Standard Brands, Inc., Type 3 yeast extract.
[3] The Wilson Laboratories liver fraction L.

preparations. These, when added to the basal diet at 1 per cent level, gave maximal growth, hemoglobin formation, and feathering, as can be seen from the data given in Table II. Several of our concentrates prepared from yeast extract, labeled Concentrates 27-203-A, 27-225-A-II, and 27-247-D, which are equivalent preparations, constantly gave maximal effects when fed at levels of 14 to 16 mg. per 100 gm. of diet. These prepa-

TABLE II

Response of Chicks to Control Preparations

Basal diet plus	No. of experiments	No. of groups	No. of birds	Survival	Weight, 4 wks.		Hemoglobin, 4 wks., per 100 cc. blood		Feather value	Potential folic acid activity,* per 100 gm. diet	
					Average	Range	Average	Range		L.c.†	S.l. R†
				per cent	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	γ	γ
No addition	16	17	168	33.7	84.7	55–108	4.53	1.8–6.5	7		
0.5% yeast extract	10	10	98	84.4	179.9	131–272	7.46	6.0–9.1	45	26	27.5
1.0% " "	12	16	160	98.1	290.9	248–333	8.9	8.2–9.4	77	52	55
2.0% " "	4	4	40	97.5	299.7	268–313	8.75	8.5–9.1	85	104	110
1.0% liver fraction L	2	2	20	95	288.2	271–306	8.6‡		70‡		
8 mg.% yeast Concentrate 27-247-D or equivalent preparation	4	7	70	93	202.5	165–235	7.12	5.7–7.6	49	22	19
16 mg.% yeast Concentrate 27-247-D or equivalent preparation	3	3	30	93.3	266.7	236–298	8.53	8.1–9.0	70	44	38

* Microbiological data obtained by the Vitamin Laboratory, E. R. Squibb and Sons, through the courtesy of Dr. A. Black.

† *Lactobacillus casei* ε activity; *Streptococcus lactis* R activity.

‡ One value only.

rations were therefore frequently used as supplements for the control groups, since they had the advantage of being free of many materials contaminating the original yeast extract. Because of the wide variations which have been reported in responses to purified basal diets used in this type of experiment, it seems that a presentation of our experience with the basal diet and with control preparations fed in adequate amounts such as are given in Table II is important, since it will help the reader to evaluate our

data on pure preparations more critically and to fit them into a self-consistent pattern.

While we were occupied with attempts to prepare concentrates of factors needed to supplement our basal diet, the first report of Scott, Norris, Heuser, Bruce, Coover, Bellamy, and Gunsalus (13) appeared, indicating that pyridoxic acids played a rôle in hemoglobin formation in chicks.   Therefore, we decided to test these materials under our experimental conditions, which were somewhat different from those used by the Cornell workers. We first made two different preparations of oxidized pyridoxine and found them to be without effect on growth or hemoglobin formation in chicks maintained on our basal diet.   We then were able to secure samples of 5-pyridoxic acid and 4-pyridoxic acid[4] which were used in place of the cruder oxidized pyridoxine preparations.

In testing the value of the 5-pyridoxic acid as a supplement to the basal diet, we added it in one experiment at a level of 2 mg. per 100 gm. of diet as the sole supplement, and in a second experiment at a level of 25 γ per 100 gm. of diet alone and in combination with various levels of a purified yeast concentrate (No. 27-247-D).   This latter preparation was known to induce optimal or near optimal responses at a level of 16 mg. per 100 gm. of basal diet. Our findings are given in Table III.

Our results were greatly at variance with those reported by Scott et al. (13), in that we observed no supplemental effects from the 5-pyridoxic acid either alone or in combination with suboptimal levels of the yeast concentrate.   The differences seemed to be resolved, however, by the subsequent publication of Scott, Norris, Heuser, and Bruce (14), in which they reported that pyridoxic acid was active only in the presence of LCF and that previous results had been due to the inclusion of factor S in their diet, which apparently contained adequate amounts of LCF.   These workers presented evidence that pure LCF of Hutchings et al. (12) plus pyridoxic acid prevented anemia and stimulated growth in chicks receiving a purified diet devoid of factor S supplement.

Soon thereafter we were able to obtain small amounts of LCF (85 per cent pure)[5] and decided to find out whether the observations of the Cornell workers could be duplicated under our experimental conditions.   Since we had only a very small amount of LCF, it was fed to smaller groups of birds than was usual and at the level of 50 γ[6] per cent alone and in combina-

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[4] Samples of 4- and 5-pyridoxic acids were kindly supplied by Merck and Company, Inc., Rahway, New Jersey, through the courtesy of Dr. Karl Folkers.

[5] The sample of LCF (85 per cent pure) was kindly furnished us by Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc., Pearl River, New York.

[6] The level of 50 γ per cent represents the level of pure material supplied when 59 γ of the 85 per cent pure sample which we had available were fed.

tion with 100 γ per cent of 4-pyridoxic acid. In this experiment we also included the 4- and 5-pyridoxic acids alone and in combination with a half optimal level of yeast concentrate, No. 27-247-D. Our results are given in Tables IV and V. Table IV shows the data on growth, survival, and hemoglobin. Table V is concerned with details of hematological studies of the same birds, and similar data taken from the literature are included for comparison.

TABLE III

Results Obtained with 5-Pyridoxic Acid and Yeast Concentrate 27-247-D*

Experiment No.	Supplement per 100 gm. diet	Average weight		Hemoglobin, 4 wks.
		3 wks.	4 wks.	
		gm.	*gm.*	*gm. per 100 cc. blood*
19-18	None	77 (7)†	74 (4)	4.1 (4)
	Yeast extract, 0.5 gm.	140 (10)	193 (9)	8.2 (9)
	" " 1.0 "	182 (10)	248 (10)	9.0 (10)
	5-Pyridoxic acid, 2.0 mg.	71 (8)	71 (2)	5.9 (1)
19-20	None	80 (5)		4.3 (5)‡
	Yeast extract, 0.5 gm.	128 (9)	176 (9)	7.7 (9)
	" " 1.0 "	185 (10)	269 (10)	8.7 (10)
	5-Pyridoxic acid, 25 γ	77 (4)		5.0 (4)‡
	Yeast Concentrate 27-247-D, 4 mg.	105 (9)	111 (7)	4.8 (7)
	" " 27-247-D, 8 "	127 (10)	165 (9)	5.7 (9)
	" " 27-247-D, 16 mg.	154 (9)	236 (9)	8.5 (9)
	" " 27-247-D, 4 mg. + 5-pyridoxic acid, 25 γ	109 (9)	105 (5)	3.8 (5)
	Yeast Concentrate 27-247-D, 8 mg. + 5-pyridoxic acid, 25 γ	144 (9)	188 (8)	6.2 (8)

* Ten birds started in each group.

† The numbers in parentheses indicate surviving birds on which the average is based.

‡ 3 weeks data.

On the basis of these findings, it seems clear that neither of the pyridoxic acids has any supplementary value when added to the basal diet alone. Neither did they supplement the yeast concentrate when the latter was fed at 8 mg. per cent, an amount which gives half optimal responses in growth and hemoglobin formation. Also, it can be seen that LCF alone, at the level fed, had a small but distinct effect on growth and survival was very good, but its effect at this level on hemoglobin formation seems to be negligible. It would have been desirable to have fed larger amounts of LCF, but we were unable to do this because of our limited supply. If one assumes that LCF is the only deficiency of our basal diet, it would

appear that it is needed in amounts greater than 200 γ per 100 gm. of diet, since 50 γ gave not more than one-quarter of the response which can be expected from other supplements such as yeast concentrates and liver fraction L.

On the other hand, it is quite evident that the combination of LCF and 4-pyridoxic acid has a significant supplemental effect in so far as growth and hemoglobin formation are concerned, although the response obtained with the combination is still very much below the optimum. In general this

TABLE IV

Effect of Lactobacillus casei ε Factor (LCF), Pyridoxic Acids, and Yeast Concentrate on Growth and Hemoglobin Formation

Supplement per 100 gm. diet	No. of birds started	Average weight			Hemoglobin, per 100 cc. blood	
		3 wks.	4 wks.	5 wks.	4 wks.	5 wks.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
None	10	97 (7)*	88 (3)		6.5 (3)	
4-Pyridoxic acid, 100 γ	10	94 (7)	104 (4)		5.1 (4)	
" " 500 "	10	92 (5)	88 (2)		2.9 (2)	
5-Pyridoxic " 100 "	10	92 (6)	134 (1)		5.1 (1)	
" " 500 "	10	110 (4)	102 (2)		5.8 (2)	
LCF, 50 γ	6	111 (6)	130 (5)		3.5 (5)	
" 50 " + 4-pyridoxic acid, 100 γ	6	160 (6)	220 (6)	265 (6)	7.7 (6)	7.7 (5)
Yeast Concentrate 27-247-D, 8 mg.	10	171 (10)	230 (10)	312 (8)	7.3 (10)	8.5 (7)
Yeast Concentrate 27-247-D, 8 mg. + 4-pyridoxic acid, 100 γ	10	152 (10)	201 (10)	277 (8)	6.7 (10)	6.8 (8)
Yeast Concentrate 27-247-D, 8 mg. + 5-pyridoxic acid, 100 γ	10	170 (10)	235 (10)	306 (9)	7.2 (10)	8.2 (9)

* The numbers in parentheses indicate surviving birds upon which the averages are based.

confirms the report of the Cornell workers (14) regarding the value of pyridoxic acid as a supplement along with LCF, but there still remain important differences between their data and ours which will be seen from a study of Table V.

In analyzing the data from the different laboratories given in Table V it is well to recognize the following facts. Under our experimental conditions and on our basal diet a more severe anemia and poorer survival are experienced than are indicated by the data of the Cornell workers (14) or the Parke, Davis investigators (10, 11). The data of Scott *et al.* (14) are for

<p style="text-align:center">TABLE V</p>

Effect of Lactobacillus casei є Factor (LCF), Pyridoxic Acids, Yeast Concentrate, and Vitamin B$_c$ on Red Blood Cell Characteristics, including Data from Literature

Supplement per 100 gm. diet	Values at 4 wks.			Calculated constants		
	Hemoglobin	Hematocrit	Red cells	Average cell volume	Average hemoglobin per cell	Average hemoglobin per 100 cc. packed cells

Authors' data; *cf.* Table III

	gm. per 100 cc. blood	per cent	10⁶ per c.mm.	cu. microns	micromicrograms	gm.
None	6.5 (3)*	14.7	1.48	99	44	44
100 γ 4-pyridoxic acid	5.1 (4)	14.1	0.77	183	66	36
500 " " "	2.9 (2)	7.3	0.68	107	43	40
100 " 5-pyridoxic "	5.1 (1)	14.5	0.82	177	62	35
500 " " "	5.8 (2)	17.5	1.24	141	47	47
Average	5.2	13.7	1.02	134	51	38
50 γ LCF	3.5 (5)	11.0	0.61	180	58	32
50 " " + 100 γ 4-pyridoxic acid	7.7 (6)	25.3	2.04	124	38	31
8 mg. yeast Concentrate 27-247-D	7.3 (10)	28.5	1.97	145	37	26
8 mg. yeast concentrate + 100 γ 4-pyridoxic acid	6.7 (10)	22.9	1.59	144	42	29
8 mg. yeast concentrate + 100 γ 5-pyridoxic acid	7.2 (10)	25.1	1.63	154	44	29

Taken from data of Scott *et al.* (14)†‡

None	2.8	11.2	0.858	131	33	25
4-Pyridoxic acid	7.4	20.1	1.520	132	49	37
LCF	6.5	23.6	1.912	123	34	28
4-Pyridoxic acid + LCF	9.8	26.0	2.192	119	45	38
Commercial chick diet	10.0	26.7	2.214	121	45	38

Calculations from data of Campbell *et al.* (10)‡

None	3.4	17.2	0.89	193	38	20
Broiler ration	8.7	32.5	2.35	138	37	27
5 γ B$_c$	5.6	21.0	1.35	155	42	27
20 " "	5.9	31.5	1.92	164	31	19
40 " "	7.0	30.3	1.95	155	36	23
100 " "	7.6	30.1	2.01	150	38	25
400 " "	7.7	29.7	1.95	152	40	26

TABLE V—*Concluded*

Supplement per 100 gm. diet	Values at 4 wks.			Calculated constants		
	Hemoglobin	Hemato-crit	Red cells	Average cell volume	Average hemo-globin per cell	Average hemo-globin per 100 cc. packed cells
Campbell *et al.* (11)‡						
	gm. per 100 cc. blood	*per cent*	*10⁶ per c.mm.*	*cu. mi-crons*	*micromi-crograms*	*gm.*
None	3.4	11.3	0.70	161	48	30
Broiler ration	7.7	31.2	2.27	138	34	25
1 γ B_c per day, by pipette	5.3	22.1	1.46	147	39	27
4 " " " " " "	6.3	24.4	1.63	150	39	26
20 " " " " " "	8.8	33.0	2.25	147	39	27

* The numbers in parentheses indicate surviving birds upon which data are based. *Cf.* Table IV.

† 3 weeks data.

‡ Red cell characteristics calculated by us from hematological data.

3 week-old birds and are representative, not average data, as is the case for all other values given in Table V. In addition, Scott *et al.* do not make clear whether their hematological study was carried out on birds getting 25 or 50 γ per cent, although it seems that the latter level is the one involved. Scott *et al.* used 1 per cent sulfasuxidine in their basal diet, which was not used by the Parke, Davis workers nor by us.

In our experience a good chick diet will allow white Leghorn cockerels at 4 weeks to have a hemoglobin value of 9.0, and a red cell count of 2.5 × 10⁶ cells per c.mm., which is what we have found to be the case with birds receiving adequate amounts of yeast extract or yeast concentrates as supplements for the basal diet. If we assume that the hematocrit will on the average be 32 per cent for normal birds, which is approximately correct and similar to that found by the Parke, Davis group (10, 11) and by Hogan and his students (9), the "normal" red cell characteristics which can be used for comparison are a volume per average cell of 128 cu. microns, an average of 36.0 micromicrograms of hemoglobin per cell, and 28.1 gm. of hemoglobin per 100 cc. of packed cells.

On this basis our data indicate that in the feeding of 50 γ per cent of LCF alone a macrocytic and hyperchromic anemia develops. The addition of 100 γ of 4-pyridoxic acid to the diet containing 50 γ of LCF brings the cell size and average hemoglobin per cell or unit of cells down into the "normal" range, even though an anemia exists as judged by hemoglobin and red cell count. On the other hand, 8 mg. of yeast concentrate per 100 gm. of diet, which is the half optimal level, allows a macrocytic condition to develop,

regardless of whether pyridoxic acids are present. In these groups receiving the yeast concentrate the hemoglobin per cell or unit of cells is within the "normal" range, but the anemia which exists is further characterized by a low cell count. The addition of pyridoxic acids to the yeast concentrate does not have a beneficial effect, but may actually depress the red cell count slightly.

Since pyridoxic acids showed no supplementary value for the birds receiving 8 mg. per cent of yeast concentrate, it seems that their effect on the basal diet alone would be negligible, and thus we may be permitted to discuss the data from the groups receiving pyridoxic acids alone along with those from the basal group; i.e., the first five groups recorded in Table V. These data indicate that on the average a somewhat macrocytic, hyperchromic condition is shown by surviving birds on the basal diet. The data are variable and not nearly as consistent as for the groups receiving even suboptimal levels of effective supplements. This is probably due to the severity of the deficiency as seen at 4 weeks under our experimental conditions. The apparent microcytic condition shown by the first group (basal group) is undoubtedly due to the fact that two of the surviving birds were sick, and showed some hemoconcentration.

The calculations made from the data of Campbell et al. (10, 11) indicate that the macrocytic anemia shown by their basal group was not entirely corrected by even the highest levels of vitamin B_c which were fed, namely 100 to 400 γ per 100 gm. of diet. This is interesting in view of the striking effect of the combination of LCF and 4-pyridoxic acid in allowing the formation of normocytic cells, as shown by our data.

In view of the data presented here on the chick activity of certain pure microbiologically active compounds, it might be of interest briefly to note from Table II that we have always been able to obtain optimal chick responses with crude or purified preparations when fed at levels which supplied 50 γ or less of vitamin B_c activity, as measured by the total microbiological activity after release of all "bound" materials by means of enzyme treatment. It is also true that the microbiologically active substances in our preparations after enzyme treatment were equally active for both Lactobacillus casei ϵ and for Streptococcus lactis R (S. faecalis R), which would point to the fact that we were probably dealing with true vitamin B_c activity and not with any appreciable amount of LCF activity. If this were true, it might explain why we were not able to find a supplementary effect of pyridoxic acid with our yeast preparations.

In one experiment not included in the tables we were able to obtain maximal growth, nearly maximal hemoglobin formation, and complete survival by feeding two highly purified preparations, one of which furnished a total of 22 γ of vitamin B_c activity per 100 gm. of diet, while the

other furnished a total of 37 γ per 100 gm. of diet. These data would in-
dicate that the required level of vitamin B$_c$ activity for chicks is probably
less than 50 γ per cent. Since most other investigators using pure materials
have found the required level to be higher, and even with amounts as high
as 100 to 400 γ per cent have not succeeded in duplicating normal responses,
our data might be taken to indicate that factors other than vitamin B$_c$,
LCF, and pyridoxic acid are required by chicks, or at least can substitute
for these compounds.

DISCUSSION

The status of the rôle of new and unidentified B complex vitamins of the
group loosely called "folic acids" in the nutrition of the chick is still con-
fused. Although the isolation of crystalline vitamin B$_c$, B$_c$ conjugate, and
LCF, and synthesis of vitamin B$_c$ have made these pure materials avail-
able to some workers for thorough investigation, reports on the use of these
compounds in chick nutrition have been few and leave a number of ques-
tions unanswered. The Parke, Davis workers (2, 10, 11) have never
obtained as good growth as might be expected from 4 week-old white Leg-
horn chicks, and in most instances they have not been able to obtain as
good hemoglobin values when feeding vitamin B$_c$ as they obtained with
their positive control groups. The statement by Hutchings et al. (12)
that LCF is chick-active is only partially true, since apparently adequate
levels gave us and the Cornell workers (14) only small responses. In
addition the Wisconsin and Cornell investigators (7, 13–16) still maintain
that factors other than vitamin B$_c$ or LCF are required by chicks, these
factors being factor R, vitamins B$_{10}$ and B$_{11}$, and pyridoxic acids.

Some of these discrepancies may be due to differences in basal diets used
by different workers, and by the improper use of microbiological data
obtained on crude preparations.

This is emphasized by the recent report by Boutwell, Geyer, Elvehjem,
and Hart (17), in which it is shown that rat requirements for B vitamins
are markedly altered by the changes in the type of fat or carbohydrate
offered the animals. The fact that vitamin B$_c$ and B$_c$ conjugate exist
together and may be contaminated with LCF or SLR makes interpretation
of early microbiological data, as used in conjunction with chick experiments,
somewhat questionable. However, it may also prove to be true that a
number of compounds of different chemical structure or certain combination
of compounds may be mutually equivalent or interchangeable. Such seems
to be the case with vitamin B$_c$ and B$_c$ conjugate, and with the combination
of LCF and pyridoxic acid as substitutes for vitamin B$_c$ activity.

Our data indicate that a severe deficiency can be produced in chickens
on a basal diet devoid of sulfonamides and lower in protein than that used

by the Parke, Davis or Cornell workers (2, 3, 10–13). Our basal diet is also devoid of p-aminobenzoic acid, which is unnecessary for adequate nutrition of chicks, as our data and those of the Wisconsin workers indicate. Severe anemia, lack of growth, and poor survival result from the deficiency, which can be completely alleviated by the addition of 1 per cent of yeast extract or 1 per cent liver fraction L, or by concentrates prepared from these raw materials. Under our conditions an amount of vitamin B_c activity equal to 50 γ or less of vitamin B_c per 100 gm. of diet seems adequate. In some cases as little as 22 to 37 γ of potential B_c activity have given us excellent growth and good hemoglobin formation.

The data of Briggs, Luckey, Mills, Elvehjem, and Hart (18) and those of Scott et al. (13, 14) indicate, as do our data, that optimal growth cannot be obtained by feeding supposedly adequate levels of either folic acid of Williams, or LCF of Hutchings et al. Furthermore, the data of Briggs et al. (18) indicate that 0.5 per cent sulfasuxidine in the basal diet almost completely voids the response in growth due to folic acid, but that sulfasuxidine does not inhibit a response to their liver concentrates which are said to be active in vitamins B_{10} and B_{11}. We also have found (unpublished data) that 0.5 per cent sulfasuxidine does not affect response to yeast extract or concentrates prepared from it. Thus our work would seem to indicate, as do the reports of the Wisconsin group, that there may be other compounds, low in potential vitamin B_c activity for microorganisms, which are highly active for chicks and which are not affected by the presence of sulfasuxidine in the diet. However, contrary to the experience of the Wisconsin group, we have been unable to identify clearly separate factors for growth, for hemoglobin formation, and for feathering, although in several experiments we have obtained results which indicate that there may be such separate factors.

In our experience with LCF and pyridoxic acid, we find, as did Scott et al. (14), that LCF alone does not give a good response in chicks, and that the addition of pyridoxic acid definitely complements the action of LCF, even though LCF is more active for chicks alone than is pyridoxic acid, as measured by survival and growth. We have found that 50 γ of LCF per 100 gm. of diet give at best only about one-fourth the maximal response which can be obtained. The response in growth and hemoglobin formation of the group receiving 50 γ of LCF and 100 γ of 4-pyridoxic acid was equal to that obtained from 8 mg. of our preparation, Concentrate 27-247-D (yeast concentrate), which indicates that the combination of these materials gives only about one-half the maximal response which can be obtained consistently under our experimental conditions. Thus we fail to find that this combination of LCF and pyridoxic acid gives a normal hemoglobin value.

Neither 4- nor 5-pyridoxic acid complemented the response obtained from 8 mg. per cent of yeast concentrate, which indicates to us that their action is entirely bound up with LCF. In this connection it should be remembered that the microbiological tests on Concentrate 27-247-D (yeast concentrate) indicated that whatever so called folic acid activity was present was probably that of vitamin B_c activity and not LCF activity, since equal response was obtained by both test organisms, *Lactobacillus casei* ε and *Streptococcus lactis* R. This observation thus confirms that of Luckey *et al.* (19), in that pyridoxic acid did not give any response when added to their liver preparations, and probably explains the results of both of us in that in both cases factors other than LCF were used as supplements and that for these factors pyridoxic acid is not complementary. These results likewise suggest a fundamental difference in the chemical nature of LCF and vitamin B_c.

In general our data show much greater variation in the groups for the calculated constants such as average cell volume, average hemoglobin per cell, and hemoglobin per 100 cc. of packed cells, than did those reported by Scott *et al.* (14), and are more in conformity with those reported by Campbell *et al.* (10, 11). Thus in order to interpret the effects of various materials on the blood picture, it is evident that more data than just hemoglobin values are necessary, and further detailed studies of the morphology and hemoglobin content of red blood cells seem likely to define some specific pathology involved in the deficiency described in this report.

SUMMARY

1. In agreement with the results of others, we have found that a purified diet containing all the known nutritive factors, other than "folic acid," regularly produces a severe deficiency in growing chicks. This deficiency is characterized by very poor growth, poor feathering, a distinct anemia, and high mortality at 4 weeks. We have not found it necessary to include sulfonamides in the basal diet to induce these deficiency symptoms.

2. Yeast extract or liver fraction L, at levels of 1 per cent, or concentrates prepared from the yeast extract, in amounts furnishing 22 to 44 γ of vitamin B_c activity as determined by microbiological assay, completely prevent the deficiency symptoms and render the basal diet complete for growing chicks.

3. Neither 4- nor 5-pyridoxic acid alone improves the performance of the chicks on the basal ration; *Lactobacillus casei* ε factor (LCF) from fermentation products at a level of 50 γ per cent has only a slight supplementary effect; but the combination of LCF and 4-pyridoxic acid induces a distinct response in growth and hemoglobin formation. This response is much less than that obtained from an equal or lesser amount of vitamin

B_c activity in the natural products. This suggests that other compounds of different microbiological activity may have biological activity for chicks on this basal diet.

4. A study of the anemia produced by the basal diet indicates that the red blood cells are macrocytic and hyperchromic, a condition which is corrected by the combination of LCF and 4-pyridoxic acid or by the yeast concentrates.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

I. THE STRUCTURE OF Δ11-LITHOCHOLENIC ACID*

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In studying methods for the synthesis of steroids with an oxygen function at carbon atom 11, Press and Reichstein (1) and McKenzie, McGuckin, and Kendall (2) prepared lithocholenic acid by pyrolysis of derivatives of desoxycholic acid. At the time this present investigation was undertaken, the publications of the Swiss investigators were unavailable to us and the work reported was undertaken to provide evidence for the position of the double bond in this substance. It was known from the investigations of Kendall and coworkers (2) that catalytic reduction of the unsaturated acid yielded lithocholic acid, which proved that the carbon skeleton was unaltered, that the double bond was not in a hindered position, and that the hydroxyl group at C-3 was not changed.

By ozonolysis and subsequent oxidation with hydrogen peroxide it was possible to convert the methyl ester acetate of lithocholenic acid (I) to the hitherto undescribed 24-methyl-3(α)-acetoxydesoxybilianic-11,12,24 acid (II). The identity of this compound was further established by oxidation with nitric acid to the known (3) choloidanic acid (III) (3∥4,11∥12-cholane-penta acid-3,4,11,12,24). These reactions are summarized in the accompanying formulas. These results prove that the position of the double bond is between C-11 and C-12 of the steroid nucleus.

This conclusion has been amply substantiated by the results of Burckhardt and Reichstein (4) and of Press and Reichstein (1) and by the work of Kendall and his associates (2, 5). Since the 11,12,24-tricarboxylic acid may be of interest in other investigations, a report of the results is made at this time.

EXPERIMENTAL[1]

24-Methyl-3(α)-acetoxydesoxybilianic-11,12,24 Acid (II)—461 mg. of methyl 3(α)-acetoxy-Δ11-lithocholenate (m.p. 118°) were dissolved in 175

ml. of dry chloroform and a 6 per cent ozone stream bubbled through the solution for 1 hour at room temperature. An aqueous solution of 5 per cent H_2O_2 was added, and the mixture shaken intermittently for 5 hours and allowed to stand at room temperature overnight. The chloroform was washed four times with water and then extracted five times with 5 per cent Na_2CO_3 solution. The Na_2CO_3 extracts were acidified immediately with chilled HCl and the acids extracted with chloroform. After drying over Na_2SO_4, the chloroform was evaporated to dryness. The product crystallized from ethyl acetate-petroleum ether as clusters of fine needles, m.p. 204–206°. The first crop weighed 140 mg. and no attempt was made

to recover further material. After two recrystallizations from dilute acetone the compound melted at 212–213°; $[\alpha]_D^{25} = +51.5°$ (CHCl$_3$).

$C_{27}H_{42}O_8$

Calculated.　C 65.56, H 8.56, neutral equivalent 247, saponification equivalent 123
Found.　　　" 65.68, " 8.60,　"　　　"　　　249,　　　"　　　　　　"　　　127

3(α)-Hydroxydesoxybilianic-11,12,24 Acid—218 mg. of the ester (II) were saponified with 2 N NaOH at room temperature for 30 minutes. The solution was acidified under ether and extracted thoroughly with ether, and the ether was washed with water. Evaporation of the solvent yielded an oil which crystallized upon addition of ethyl acetate. The product was recrystallized from ether-ethyl acetate as clusters of small needles, m.p. 259–262°; $[\alpha]_D^{27} = +53.5°$ (absolute ethanol).

$C_{24}H_{38}O_7$. Calculated, C 65.73, H 8.73; found, C 65.52, H 8.65

Choloidanic Acid (III) from 24-Methyl-3(α)-acetoxydesoxybilianic-11,12,-24 Acid (II)—110 mg. of II were mixed with 2.0 ml. of HNO$_3$ (sp. gr. 1.40) and warmed gently on the hot-plate. Within a few minutes a relatively vigorous reaction occurred and a mass of fine crystals formed. The reac-

tion was completed by gentle warming on the steam bath for $1\frac{1}{2}$ hours and the crystals filtered off and washed. After one recrystallization from dilute acetic acid the compound melted at 314° and gave no depression when mixed with an authentic specimen of choloidanic acid (m.p. 311°) prepared from dehydrodesoxycholic acid. The higher melting point of the choloidanic acid from II is a more correct value, since a prolonged HNO_3 oxidation is necessary in the case of the preparation of the product from dehydrodesoxycholic acid.

SUMMARY

The position of the double bond in Δ^{11}-lithocholenic acid was demonstrated by oxidation to the new compound $3(\alpha)$-hydroxydesoxybilianic-11,-12,24 acid and the oxidation by nitric acid of this substance to the known choloidanic acid.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

II. SOME REACTIONS OF AN EPOXIDE OF Δ11-LITHOCHOLENIC ACID*

By T. F. GALLAGHER and WILLIAM P. LONG†

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The preparation of Δ11-lithocholenic acid by Kendall and coworkers (1) and by Press and Reichstein (2) yields an important substance for the partial synthesis of steroid compounds related to adrenal cortical hormones. We have investigated certain reactions of the epoxide of this compound, since it seemed desirable to explore the possibility of introducing an oxygen function at C-11 of the steroid nucleus by way of this derivative.

Both Kendall (1) and Press and Reichstein (2) have described an 11,12 epoxide prepared by the action of perbenzoic acid on Δ11-lithocholenic acid or its derivatives. Kendall did not assign configuration to the epoxide. Press and Reichstein (2) designated the product $11(\beta),12(\beta)$-epoxy because of the fact that desoxycholic acid (in which the hydroxyl group at C-12 has β configuration according to Reichstein and Sorkin (3)) can be isolated when the epoxide is reduced by hydrogen in the presence of Raney nickel under 150 atmospheres at 100°. Since the configuration of the hydroxyl group at C-12 in desoxycholic acid is unsettled (4), it seemed undesirable to assign a definite configuration to the epoxide on the basis of these results.

Kendall[1] in earlier work had noted that when $3(\alpha)$-hydroxy-11,12-epoxy-cholanic acid (1) was dissolved in acetic acid there was an immediate positive shift in rotation upon the addition of hydrobromic acid. Since the action of mineral acid on epoxides can lead to many compounds, depending upon the nature of the acid, the solvent, temperature, and other variables, it seemed desirable to investigate the reaction under precisely defined conditions. For the purpose of following qualitatively the course of reaction we have made use of the considerable changes in optical rotation which accompany the transformations of the epoxide. Although not all of the possible products have been identified, our experiments indicate that the principal chemical changes involved when an acetone or acetic acid solution of the

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[1] Kendall, E. C., personal communication.

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11,12 epoxide is treated with hydrobromic or sulfuric acid are the intro-
duction of the anionic constituent at C-11 and the formation of a hydroxyl
group at C-12.   The stability of the substituent at C-11 is, however,
markedly influenced by the experimental conditions.

<center>EXPERIMENTAL[2]</center>

Methyl 3(α)-Acetoxy-11,12-epoxycholanate $(I, R_1 = CH_3—C—; R_2 =$
$CH_3)$—This was prepared by treating a chloroform solution of methyl 3(α)-
acetoxy-Δ^{11}-cholenate (m.p. 118–120°) with excess perbenzoic acid in

$$R_1 = H \text{ OR } CH_3-C— \qquad R_2 = H \text{ OR } CH_3$$

chloroform at room temperature.   The reaction was rapid and quantita-
tive.   The chloroform was washed with sodium carbonate solution and
with water, and, after drying with Na_2SO_4, the solvent was removed in a
partial vacuum.   Crystallized from acetone the epoxide formed prisms
melting at 145°; $[\alpha]_D^{22} = +57°$ (acetone or acetic acid); $[\alpha]_D^{23} = +26°$
(toluene).

$C_{27}H_{42}O_5$.   Calculated, C 72.61, H 9.48; found (T. S. M.), C 72.49, H 9.17

Methyl 3(α)-Hydroxy-11,12-epoxycholanate (I, $R_1 = H$; $R_2 = CH_3$)—This was prepared in a similar fashion to that of the previous compound from methyl 3(α)-hydroxy-Δ^{11}-cholenate (m.p. 101–103°). Upon recrystallization from petroleum ether and from dilute methanol it formed clusters of small plates which melted at 101–103°; $[\alpha]_D^{21} = +44°$ (acetone or acetic acid); $+12°$ (toluene).

3(α)-Hydroxy-11,12-epoxycholanic Acid (I, R_1 and $R_2 = H$)—This was prepared by saponification at room temperature with aqueous alcoholic KOH of either of the two preceding products. After recrystallization from ethyl acetate-petroleum ether, the compound formed needles, m.p. 164–165°; $[\alpha]_D^{25} = +44°$ (acetic acid); $+40.2°$ (acetone or ethanol); $+20.5°$ (toluene).

Methyl 3(α)-Acetoxy-11(β)-bromo-12(α)-hydroxycholanate (II, $R_1 =$

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-;\ R_2 = CH_3)$$—1.109 gm. of methyl 3(α)-acetoxy-11,12-epoxycholanate were dissolved in 20 ml. of redistilled acetone. 5.0 ml. of 0.72 N hydrobromic acid in acetone were added and the mixture allowed to stand at room temperature for 15 minutes. In this experiment the rotation was not measured but in a number of other instances the specific rotation (calculated from the weight of oxide taken for the determination[3]) immediately rose to +80°. The acetone solution was diluted with a relatively large amount of ether and washed six times with water. The combined water washings were titrated with 0.1 N alkali to the phenolphthalein end-point and 13.0 ml. were consumed. The reaction of the oxide with HBr used, therefore, 2.3 ml. of N HBr or 92 per cent of the theoretical amount. This is in agreement with other experiments in which the acetone solution was directly titrated with standard base. The ether was removed at room temperature, and the residue dried in an evacuated desiccator over P_2O_5. Upon crystallization from a mixture of ether and

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[3] The marked dextrorotatory shift when the epoxide reacts with HBr in either acetone or acetic acid solution is probably due to the simultaneous formation of small amounts of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid or its derivatives, including the 12-acetoxy derivative, since the observed rotation of the bromohydrin is too small to account for the increase. This explanation is consistent both with the isolation of this compound from the reaction in acetic acid in the presence of H_2SO_4 and with the fact that the amount of HBr which reacts with the epoxide in either acetone or acetic acid is slightly less than 95 per cent of the theoretical amount. The fact that this side reaction takes place to a lesser extent in acetone than in acetic acid, measured by change in rotation, is to be ascribed to the greater acid strength of HBr in acetic acid. In agreement with this explanation, increasing the concentration of H_2SO_4 in acetic acid should markedly accelerate this reaction, a fact which is in accord with the observed changes in rotation.

60–70° petroleum ether the compound formed clusters of prisms, m.p. 137.5–139°; $[\alpha]_D^{22} = +54.2°$ (absolute ethanol).

$C_{27}H_{43}O_5Br$. Calculated, C 61.47, H 8.26; found (A. M.), C 61.60, H 8.36

When the bromohydrin, dissolved in acetone, was titrated with aqueous alkali, the theoretical amount of base was consumed and the epoxide re-formed. The product was identical with the starting material and gave no depression of melting point on admixture.

$3(\alpha),12(\alpha)$-*Dihydroxy-11*(β)-*bromocholanic Acid* (II, R_1 and $R_2 = H$)— 515.6 mg. of $3(\alpha)$-hydroxy-11,12-epoxycholanic acid were dissolved in 49 ml. of acetone, 1.0 ml. of 48 per cent HBr added, and the mixture allowed to stand 20 minutes at room temperature. The solution was poured into ether and extracted thoroughly with water. After drying over Na_2SO_4 the ether was removed at room temperature under diminished pressure. The product crystallized from a mixture of ether and 90–100° petroleum ether. After four recrystallizations from ethyl acetate-petroleum ether, the bromohydrin formed clusters of prisms which melted sharply at 142.5°; $[\alpha]_D^{21} = +54.2°$ (absolute ethanol).

$C_{24}H_{39}O_4Br$. Calculated, C 61.14, H 8.34, Br 16.95; found,[4] C 61.04, H 8.30, Br 16.74

Methyl $3(\alpha)$-*Acetoxy-11*(β)-*bromo-12-ketocholanate* (III, $R_1 = CH_3\!\!-\!\!\overset{\displaystyle O}{\overset{\|}{C}}\!\!-$; $R_2 = CH_3$)—210 mg. of the bromohydrin (methyl $3(\alpha)$-acetoxy-11(β)-bromo-12(α)-hydroxycholanate) were dissolved in 10 ml. of stable glacial acetic acid and 3.0 ml. of 0.56 N CrO_3 in acetic acid added. The reaction mixture stood at room temperature for 4 hours and the excess CrO_3 was reduced by the addition of methanol. The mixture was poured into ether and extracted thoroughly with water, sodium carbonate, dilute hydrochloric acid, and finally with water. Upon evaporation of the ether solution under diminished pressure at a temperature not exceeding 35°, the product crystallized. It was recrystallized from an ethyl acetate-petroleum ether mixture as well formed needles, m.p. 162–164°; $[\alpha]_D^{20} = +41.4°$ (absolute ethanol).

$C_{27}H_{41}O_5Br$. Calculated.   C 61.71, H 7.86, Br 15.21
Found (T. S. M.). " 61.96, " 7.82, " 14.80

Methyl $3(\alpha)$-*Acetoxy-12-ketocholanate* (IV, $R_1 = CH_3\!\!-\!\!\overset{\displaystyle O}{\overset{\|}{C}}\!\!-$; $R_2 = CH_3$) —71 mg. of methyl $3(\alpha)$-acetoxy-11(β)-bromo-12-ketocholanate were dissolved in 2.5 ml. of glacial acetic acid and boiled for 1 hour under a reflux

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[4] Microanalysis by Dr. Joseph Alicino, The Squibb Institute for Medical Research.

with 50 mg. of zinc dust. After filtration the solution was diluted with ether and extracted thoroughly with sodium carbonate solution and with water. The ether was removed and the product crystallized from dilute ethanol. After one recrystallization it melted at 145–147°. Upon admixture with an authentic specimen of methyl 3(α)-acetoxy-12-ketocholanate (m.p. 150–151°) there was no depression of the melting point.

Reaction of 11,12 Epoxide with Concentrated H_2SO_4 in Acetone—Within a short time after the addition of H_2SO_4 to an acetone solution of the 11,12 epoxide there is an increase in specific rotation and parallel with this a decrease in titratable acidity (Fig. 1). The rotation falls progressively with time and concomitantly the titratable acidity increases. The prod-
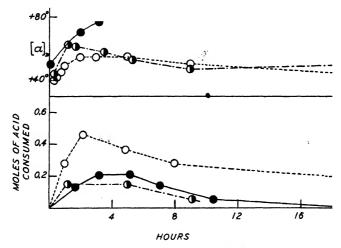
FIG. 1. Mutarotation of 11,12 epoxide in 0.07 N H_2SO_4 in acetone. ●, methyl 3(α)-acetoxy-11,12-epoxycholanate; ○, methyl 3(α)-hydroxy-11,12-epoxycholanate; ◑, 3(α)-hydroxy-11,12-epoxycholanic acid.

ucts obtained are unsaturated, as shown by titration with perbenzoic acid, and there was likewise a slight reaction with lead tetraacetate. No crystalline products were obtained.

Reaction of 11,12 Epoxide with Hydrobromic Acid in Acetic Acid—An acetic acid solution of hydrobromic acid was prepared by dissolving a carefully weighed amount of 48 per cent aqueous hydrobromic acid in glacial acetic acid. The stock solution was then diluted with glacial acetic acid to the desired molarity of HBr. The epoxide was dissolved in glacial acetic acid, slightly more than the calculated amount of the hydrobromic acid introduced, and the solution made to volume. Polariscope readings were then taken at suitable intervals thereafter until the rotation reached a constant value (Fig. 2).

Estimations of the amount of hydrobromic acid present after reaction with the epoxide were made in the following manner. A series of dilutions of hydrobromic acid was prepared equivalent to from 1.0 to 14.0 ml. of 0.01 M HBr in 10 ml. of acetic acid. o-Nitroaniline was used as the indicator and a standard reference curve of the relation between color and hydrogen ion concentration constructed from the galvanometer readings of an Evelyn photoelectric colorimeter. It was necessary to have a constant concentration of indicator per ml. of solution and, for the most accurate results, to use the range between 1.0 and 7.0 ml. of 0.01 M HBr per 10 ml. of acetic acid. The accuracy of the procedure was checked by titration of the hydrobromic acid with a standard solution of anhydrous sodium acetate in glacial acetic acid; estimations could be made with an error of approximately 3 per cent, which was adequate for the investigation.

The epoxide reacts with 95 per cent of the theoretical amount of hydro-
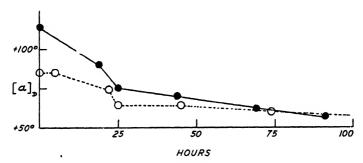
FIG. 2. Mutarotation of methyl 3(α)-acetoxy-11,12-epoxycholanate in acetic acid in the presence of a considerable molar excess of H_2SO_4 and HBr. ●, H_2SO_4; ○, HBr.

bromic acid, the reaction being identical with that in acetone. We did not attempt the isolation of the bromohydrin from this reaction, but a satisfactory proof for the initial formation of this compound was obtained by the isolation of methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate. An excess of the hydrobromic acid was added to an acetic acid solution of methyl 3(α)-acetoxy-11,12-epoxycholanate and after a few minutes an excess of CrO_3 was introduced. The reaction mixture stood at room temperature for 4 hours. When the product was isolated as before, it proved to be methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate, identical in all respects with the compound obtained by oxidation of the bromohydrin.

When methyl 3(α)-acetoxy-11,12-epoxycholanate was allowed to stand in acetic hydrobromic acid solution until the rotation had fallen to +60° (30 hours), it proved impossible to isolate a crystalline product.

Reaction of 11,12 Epoxide with Sulfuric Acid in Glacial Acetic Acid—In

the presence of excess sulfuric acid, the rotation rises immediately and within a short interval begins to fall again. This abrupt change is not accompanied by a change in acidity measured as before. Similar changes in rotation are observed with less than equimolar amounts of sulfuric acid. With less than equivalent amounts of sulfuric acid the reaction proceeds for about 2 hours, after which the specific rotation remains constant (Fig. 3). The products isolated from this experiment are nevertheless a mixture. Caution must be observed in working up the products, for, if the material is heated upon the steam bath, the oily residue becomes yellow and the specific rotation falls to a much lower value. This change can be prevented by removing the solvents under diminished pressure at room temperature.

593.3 mg. of methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate (1.33 mM) in 10 ml. of glacial acetic acid with 0.0957 mM of sulfuric acid stood at room
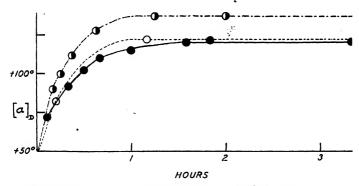
FIG. 3. Mutarotation of 11,12 epoxide in 0.01 M H_2SO_4 in acetic acid. ●, methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate with 0.28 mole of H_2SO_4 per mole of epoxide; ○, methyl $3(\alpha)$-hydroxy-11,12-epoxycholanate with 0.29 mole of H_2SO_4 per mole of epoxide; ◑, $3(\alpha)$-hydroxy-11,12-epoxycholanic acid with 0.23 mole of H_2SO_4 per mole of epoxide.

temperature for $3\frac{3}{4}$ hours. The rotation at this time was $[\alpha]_D = +120°$. The solution was poured into ether and washed twenty times with H_2O. The ether solution was dried over Na_2SO_4, the ether distilled off below 28° (bath temperature), and the oily residue dried in the presence of P_2O_5 at room temperature. 605 mg. of colorless oil, $[\alpha]_D = +119°$, were obtained. This material had a saponification equivalent of 190 after 2 hours boiling with base. It reacted with perbenzoic acid at room temperature, 48.4 mg. consuming 0.71 mg. of active oxygen in 24 hours.

These results indicated that the reaction resulted in part in an acetolysis of the epoxide catalyzed by hydrogen ion. Since by analogy to the reaction of the epoxide with HBr the acetoxy grouping should have been introduced at C-11, the mixture was carefully investigated for the presence of this compound. 5.4 gm. of methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate were

dissolved in 200 ml. of glacial acetic acid, 4.0 ml. of 0.958 M H_2SO_4 in glacial acetic acid added, and the mixture allowed to stand at room temperature for $3\frac{1}{2}$ hours. At this time the specific rotation (calculated from the epoxide) was $+114°$. The solution was poured into ether, washed with twenty successive portions of water, dried over Na_2SO_4, and the ether removed under diminished pressure at 30°. The residual oil was thoroughly dried in a desiccator, dissolved in 70 ml. of a mixture of benzene-petroleum ether, 2:3, and poured over a column of 50 gm. of activated Al_2O_3. The fractions collected are given in Table I.

$3(\alpha),12$-Dihydroxy-$\Delta^{9,11}$-cholenic Acid (V, $R_1 = H$; $R_2 = H$)—Fraction 1 was rechromatographed over 50 gm. of Al_2O_3 with petroleum ether alone as the solvent. 1000 ml. of this solvent eluted in the initial fraction 500 mg. of an oily substance; $[\alpha]_D = +180°$. Several other fractions were eluted, but, since the rotation was slightly lower, these were considered mixtures and were not further investigated. Two duplicate portions each con-

TABLE I

Chromatographic Separation of Acetolysis Products

Fraction No.	Solvent	Ratio	Volume	Weight of eluate	$[\alpha]_D$
			ml.	*gm.*	*degrees*
1	Benzene-petroleum ether	2:5	200	2.9	+158
2	" "	3:1	2000	1.4	+60
3	Ether		1300	0.6	+55
4	Methanol		150	0.2	+45

taining 200.4 mg. of the oil were saponified with 0.25 N NaOH in ethanol for 2 hours under a reflux. The saponification equivalent was 208. The neutral solution was acidified under ether, and the acid extracted with ether, washed, and evaporated to dryness. Upon solution in acetone-benzene mixture crystals deposited on standing, m.p. 168–180°; $[\alpha]_D^{22} =$ $+99.3°$. Recrystallized from ethanol the compound melted at 166–170°. Recrystallized from ethyl acetate-petroleum ether it sintered at 149° and melted at 178–184° with some preliminary softening. 2.7 mg. dissolved in 2.0 ml. of acetic acid containing a very small amount of H_2SO_4 gave a specific rotation of $+237°$ when read 8 minutes after mixing.

$C_{24}H_{38}O_4$. Calculated, C 73.81, H 9.81; found (A. M.), C 74.78, H 10.43

A sample of the compound was sent to Dr. E. C. Kendall for comparison with his compound, $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid. Dr. Kendall reported that there was no depression on admixture with an authentic specimen of $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid, and, since he and his

coworkers have reported a similar marked increase in rotation when their compound was treated with mineral acid in acetic acid solution, there can be little doubt that the two substances are identical.[5]

3(α),12(α)-Dihydroxy-11(β)-acetoxycholanic Acid (VII)—Fractions 2, 3, and 4 from the initial chromatogram were combined and chromatographed over 50 gm. of Al_2O_3. A small amount of high rotatory component was removed by elution with benzene and then 1.29 gm., $[α]_D = +45°$, were removed with 400 ml. of benzene-ether, 3:2. This material failed to crystallize under a variety of conditions. Two duplicate portions each containing 190 mg. were hydrolyzed by boiling with 7.0 ml. of 0.25 N NaOH in 50 per cent ethanol for 2 hours. The saponification equivalent was 279. The solution of the sodium salt was added to dilute H_2SO_4 dropwise with stirring at 50°. After cooling to room temperature, the crystalline product was filtered off and recrystallized from dilute ethanol. Needles melting at 148–160° were obtained. After three recrystallizations from dilute ethanol the melting behavior was unchanged. After recrystallization from ethyl acetate-petroleum ether the product formed clusters of fine needles melting at 202–230°. After four further recrystallizations from this mixture the melting point remained constant at 232–233°; $[α]_D^{20} = +52°$ (95 per cent ethanol).

$C_{26}H_{42}O_6$. Calculated, C 69.30, H 9.40; found (A. M.), C 69.15, H 9.60

34.5 mg. dissolved in 1.6 ml. of 0.624 N KOH in 30 per cent ethanol and heated under a reflux for 3 hours consumed 0.152 ml. of N base, or a saponification equivalent of 227. Calculated for $C_{26}H_{42}O_6$ (2 equivalents), 226.

Methyl 3(α)-Acetoxy-11,12-diketocholanate $(VIII, R_1 = CH_3-\overset{O}{\overset{\|}{C}}-; R_2 = CH_3)$—In subsequent experiments with larger quantities of epoxide for the acetolysis a third component of the reaction mixture was separated from the lower dextrorotatory fraction. This product was more strongly adsorbed on Al_2O_3 than methyl 3(α),11(β)-diacetoxy-12(α)-hydroxycholanate. Elution with ethyl acetate yielded an oil; $[α]_D = +45°$. 1.5 gm. of this product, dissolved in 20 ml. of stable glacial acetic acid, were oxidized with 30 ml. of 0.78 N CrO_3 in acetic acid at room temperature for 30 minutes. Excess CrO_3 was reduced with methanol, and the solution diluted with ether and extracted with water. The ether solution was washed with $NaHCO_3$ solution and with water. After drying over Na_2SO_4, the ether

[5] Dr. Kendall likewise informed us that 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid forms relatively stable choleic acids with benzene, which may account for our high carbon figure. We are grateful to Dr. Kendall for his assistance.

was removed and 910 mg. of neutral material obtained.   This was purified by chromatographing over 15 gm. of Al_2O_3.   Elution with benzene-petroleum ether, 3:1, yielded an oily fraction which deposited 180 mg. of crystals melting at 181–186°.   Further elution of the column yielded only oily products.   The compound was recrystallized from ethyl acetate-petroleum ether and after four recrystallizations had a constant melting point of 197–198°; $[\alpha]_D^{21} = +129°$ (ethyl acetate).

$C_{27}H_{40}O_6$.   Calculated, C 70.40, H 8.75; found,[4] C 70.51, 70.23, H 8.93, 8.81

DISCUSSION

The 11,12 epoxide of lithocholenic acid obtained by means of perbenzoic acid reacts with hydrobromic acid to form a bromohydrin the structure of which was 3(α),12-dihydroxy-11-bromocholanic acid.   Since the opening of an oxide ring is with few exceptions accompanied by inversion of one of the substituents, the probability is strong that in the bromohydrin the halogen atom at C-11 and the hydroxyl group at C-12 are *trans* with respect to one another.   It has been demonstrated by Kendall (1) that this same epoxide can be converted to desoxycholic acid by catalytic reduction in the presence of hydrochloric acid.   The results of the investigation reported here demonstrate the intermediate formation of the 11-halogen-12-hydroxy derivative in the reduction so that the hydroxyl group at C-12 of this halohydrin must have the same configuration as the C-12 hydroxyl group of desoxycholic acid.

On the basis of Giacomello's (5) x-ray measurements of desoxycholic acid Koechlin and Reichstein (4) have provisionally designated the C-12 hydroxyl group as β; that is, *cis* with respect to the methyl group at C-10 (formula IX).

(IX)

If this formulation is correct, then the bromohydrin described in this

paper should be designated 3(α),12(β)-dihydroxy-11(α)-bromocholanic acid with the structure X.

(X)

On the basis of work reported in Paper IV of this series (6), we had concluded that the C-12 hydroxyl group of desoxycholic acid has the α configuration and for this reason we had initially not assigned configuration to the halogen derivatives at C-11 and the hydroxyl group at C-12 in the present paper. After this manuscript had been prepared, however, we have had access to more recent publications of Reichstein and his collaborators which have necessitated a discussion of the configuration of these compounds.

Seebeck and Reichstein (7) have prepared two crystalline epimers from the bromination of 3(α)-acetoxy-12-ketocholanic acid. These were converted to the methyl esters which were described as follows:

	M.p. °C.	$\alpha]_D$ (CHCl₃) degrees
Methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate ..	160–161	+37.5
" 3(α)-acetoxy-11(α)-bromo-12-ketocholanate..	159–161	+47.3

For other purposes we had independently prepared these same two compounds by a different procedure and therefore both substances were available for comparison. While the two epimers differ only slightly in melting point and rotation, it seems unquestionable that the methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate (III) which we have described in the present communication with a melting point of 162–164° and $[\alpha]_D^{20} = +41.4°$ in absolute ethanol is identical with the 11(β)-bromo ester reported by Seebeck and Reichstein.[6]

[6] We have since verified this conclusion by dehydrobromination of the compound with pyridine. In agreement with the findings of Seebeck and Reichstein the compound is converted in excellent yield to methyl 3(α)-acetoxy-12-keto-$\Delta^{9,11}$-cholenate (m.p. 148–149°; $\epsilon_{2400} = 10,600$) after 3 hours heating. The 11(α) epimer is recovered unchanged under these conditions.

Seebeck and Reichstein assigned the β configuration to the halogen at C-11 of the compound under discussion after comparison of the ease of dehydrobromination of the two bromo ketones epimeric at C-11.   The 11(β)-bromo compound readily lost HBr to form methyl 3(α)-acetoxy-12-keto-$\Delta^{9,11}$-cholenate when it was warmed with pyridine, whereas the 11(α) compound was recovered unchanged from the same treatment. It is to be expected that a *trans* configuration of the hydrogen atom at C-9 and the halogen at C-11 would favor the removal of these substituents as HBr.   Since it is most probable (8) that the hydrogen at C-9 is *trans* to the angle methyl group at C-10, the epimer which was readily converted to the α,β-unsaturated keto acid was assigned the β configuration at C-11.   In Paper IV of this series (6) we shall offer further evidence for the validity of the configuration of the halogen at C-11.   We therefore believe that certainty can be attached to the configuration which Seebeck and Reichstein have given the two C-11 epimers of methyl 3(α)-acetoxy-11-bromo-12-ketocholanate.   For the further discussion then we shall assume that the configuration of the halogen at C-11 is established.

Therefore, if the structure of the bromohydrin obtained by treatment of the epoxide of Δ^{11}-lithocholenic acid is 3(α),12(β)-dihydroxy-11(β)-bromocholanic acid, either of two conclusions must be drawn.   These are (*a*) that the opening of the epoxide has occurred without inversion, or (*b*) that the configuration of the hydroxyl at C-12 has been incorrectly assigned by Koechlin and Reichstein (4).   The first conclusion, while not impossible, is unlikely.   It is therefore necessary to review the evidence which bears upon the second conclusion.   Koechlin and Reichstein (4) have presented valuable information in this regard.   Comparison of the velocity of saponification of the two epimeric methyl 3-keto-12-acetoxy-cholanates established the fact that the ester epimeric at C-12 was much more readily hydrolyzed than the analogous compound in which the C-12 hydroxyl had the configuration of that of desoxycholic acid.   The length of the side chain exerts considerable influence on the velocity of saponification of the ester at C-12, as shown by comparison of the cholanic acid derivatives with the corresponding etio acid (9) which is considerably more readily saponified.   These authors (4) state then, "Dieser starke Einfluss der Seitenkette wäre am besten verständlich, wenn sich die 12-ständige Hydroxylgruppe und die Methingruppe Nr. 17 in cis-Stellung zueinander befinden (also OH und Methyl Nr. 18 trans-ständig) . . ."

These authors likewise attempted to prepare a δ-lactone from bisnor-desoxycholic acid by heating it in tetralin and by vacuum distillation at 300°.   They were unsuccessful in these attempts, and, although they were unwilling to assign a definite configuration to the C-12 hydroxyl group and the side chain on the basis of a negative result, they nevertheless

indicate that their experimental findings are in agreement with the formulation of desoxycholic acid as either XI or XII in which the hydroxyl group at C-12 and the side chain at C-17 are in *trans* position to each other. Despite these results, the Swiss investigators provisionally accepted the formulation of Giacomello and have adopted the β configuration for the C-12 hydroxyl group of desoxycholic acid (XII).

(XI)

(XII)

Our experimental results are in better agreement with the conclusion that the C-12 hydroxyl group of desoxycholic acid has the α configuration and there is no contradictory evidence from Reichstein's work. The 11,12 epoxide with HBr yields a $3(\alpha),12$-dihydroxy-$11(\beta)$-bromocholanic acid which can be catalytically reduced to desoxycholic acid (1). Since the opening of the oxide should yield substituents at C-11 and C-12 in *trans* configuration to each other and since the halogen at C-11 has been shown to have the $11(\beta)$ configuration, it follows that the hydroxyl at C-12 of desoxycholic acid has α configuration. Therefore the correct structure of desoxycholic acid, $3(\alpha),12(\alpha)$-dihydroxycholanic acid, is shown in XI.

Accordingly, then, the bromohydrin obtained by opening the epoxide ring is $3(\alpha),12(\alpha)$-dihydroxy-$11(\beta)$-bromocholanic acid (II), and, since the opening of the epoxide ring should proceed in the same fashion with

acetic acid as with hydrobromic acid, the amorphous product of acetolysis should be methyl $3(\alpha),11(\beta)$-diacetoxy-$12(\alpha)$-hydroxycholanate (VI). Examination of molecular models reveals that this is the more hindered configuration at C-11, which is in accord with our finding that 2 hours hydrolysis with boiling 0.25 N NaOH yielded the crystalline $3(\alpha),12(\alpha)$-dihydroxy-$11(\beta)$-acetoxycholanic acid (VII).

The formation of both methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate (V) and the glycol, methyl $3(\alpha)$-acetoxy-11,12-dihydroxycholanate, as well as the acetolysis product, methyl $3(\alpha),11(\beta)$-diacetoxy-$12(\alpha)$-hydroxy-cholanate (VI), in the reaction of the epoxide with acetic acid in the presence of H_2SO_4 indicates the complexity of the reaction. These substances, however, represent the principal products. The formation of methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate is quite readily explained on the basis of the configuration we have assigned to the compounds formed by the fission of the epoxide ring. If the elements either of water or of sulfuric acid are added to the epoxide, the substituent at C-11 is *trans* to the hydrogen at C-9 and is more labile in this configuration. It is to be expected that the elements of water or sulfuric acid would be readily lost under the influence of the sulfuric acid present in the mixture. With higher acid concentration this reaction should take place more rapidly and completely. A strongly dextrorotatory substance, very probably methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate, was the principal reaction product in the presence of higher concentrations of strong acid. This finding is further evidence for the configuration we have assigned to the substituents at C-11.

We wish to express our appreciation of the technical assistance given us by Miss Joanna Xenos.

SUMMARY

1. The 11,12 epoxide formed from Δ^{11}-lithocholenic acid has $11(\alpha),12(\alpha)$ configuration.

2. The epoxide reacts with hydrobromic acid to form a bromohydrin in which the halogen is at C-11.

3. The configuration assigned this compound is $3(\alpha),12(\alpha)$-dihydroxy-$11(\beta)$-bromocholanic acid.

4. The configuration of the C-12 hydroxyl group of desoxycholic acid is discussed and the conclusion drawn that this has the α configuration or *trans* to the angle methyl groups at C-10 and C-13.

5. When the 11,12 epoxide of Δ^{11}-lithocholenic acid reacts with acetic acid in the presence of sulfuric acid, at least three compounds are formed.

These have been identified as $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid, $3(\alpha),11,12$-trihydroxycholanic acid, and $3(\alpha),12(\alpha)$-dihydroxy-$11(\beta)$-acetoxycholanic acid.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

III. PREPARATION OF 3(α),11(α)-DIHYDROXYCHOLANIC ACID*

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In Paper II we (1) have shown that methyl 3(α)-acetoxy-11,12-epoxycholanate undergoes fission of the epoxide ring with the introduction of the anionic constituent at C-11. The products of such reactions offered, we felt, a possible route to the preparation of 3(α),11-dihydroxycholanic acid.

Marker and Lawson (2) prepared 3(α),11-dihydroxy-12-ketocholanic acid by bromination and subsequent alkaline hydrolysis of 3(α)-acetoxy-12-ketocholanic acid. They could not reduce the product by the Clemmensen method but were able to obtain a crude semicarbazone. They state that Wolff-Kishner reduction was also unsuccessful, although no details of procedure or products were given. Longwell and Wintersteiner (3) reinvestigated the problem and were unable to secure the semicarbazone described by Marker and Lawson. These authors could not obtain either a hydrazone or an oxime and upon treatment of the 3(α),11-dihydroxy-12-ketocholanic acid with sodium ethylate and hydrazine hydrate at 200° both oxygen atoms in Ring C were eliminated. Marker, Shabica, Jones, Crooks, and Wittbecker (4) repeated the reduction of 3(α),11-dihydroxy-12-ketocholanic acid, using the method employed by Longwell and Wintersteiner, and obtained a product characterized as 3(α),11,12-trihydroxycholanic acid. Although it appeared from these results that the reduction of a 12-keto bile acid with a hydroxyl group at C-11 would not yield the desired product, there were at least two reasons for investigating this problem. It was possible (1) that the structure of the compound prepared by Marker and Lawson was not that assigned by these authors, or (2) that, if the structure were correct, the epimeric compound with the C-11 hydroxyl group in the opposed configuration would react with ketonic reagents and permit reduction of the carbonyl group. The experiments recorded here were undertaken to explore these possibilities.

Since the preparation of methyl $3(\alpha),11(\beta)$-diacetoxy-$12(\alpha)$-hydroxy-cholanate by acetolysis of the 11,12 epoxide gave poor yields, a series of experiments was performed in an attempt to improve the conditions. Methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate reacts with acetic acid at 100° in the absence of mineral acid, as measured by the change in rotation, but, since much unchanged epoxide could be recovered after 1 to 2 hours heating, it appeared that these conditions favored the formation of methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate (or the 12-acetate of this compound) and this method was abandoned in favor of the acid-catalyzed reaction described in Paper II (1).

Trial experiments in which rotation alone was measured gave the following results: When higher concentrations (0.02 M) of anhydrous perchloric acid were added to an acetic acid solution of the epoxide, the rotation increased to +170° immediately; with smaller concentrations (0.002 M) the same rotation was observed after 1 hour at room temperature. These results demonstrated that the conversion of the epoxide to the $\Delta^{9,11}$ acid was more rapid at high hydrogen ion concentration than the acetolysis. A series of experiments was carried out with small amounts of sulfuric acid (1.2×10^{-4} M), the solution of the epoxide being heated to boiling for from 10 to 20 minutes. The procedure gave relatively large amounts (50 to 60 per cent) of methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate and from 15 to 20 per cent of methyl $3(\alpha)$-acetoxy-11,12-dihydroxycholanate, while the yield of the desired methyl $3(\alpha),11(\beta)$-diacetoxy-$12(\alpha)$-hydroxycholanate was small and variable. When the reaction was studied at 0° with higher acid concentration (1.25×10^{-2} to 4.6×10^{-2} M), the formation of methyl $3(\alpha)$-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate was found to be less than when the reaction was conducted at higher temperatures. The yield of the acetolysis product was not markedly higher when calculated from the amount of epoxide used for the experiment. Since, however, some 40 to 50 per cent of unchanged oxide could be recovered after 18 hours, this appeared to be a more economical procedure and a minor modification of these conditions was used for the acetolysis. A typical procedure is described in the experimental section. These experiments were complicated by our failure to obtain methyl $3(\alpha),11(\beta)$-diacetoxy-$12(\alpha)$-hydroxycholanate in a crystalline state, and, for this reason, estimates of the yield of this compound were based on the amount of crystalline methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate obtained after chromic acid oxidation.

An alternative method for the preparation of an 11-acetoxy derivative in which methyl $3(\alpha)$-acetoxy-$11(\beta)$-bromo-12-ketocholanate was treated with potassium acetate in methanol solution yielded only unchanged

starting material and small amounts of amorphous product. The action of silver acetate on methyl 3(α)-acetoxy-11(β)-bromo-12(α)-hydroxy-cholanate was investigated. A portion of the material was converted to the 11,12 epoxide, while the remainder underwent loss of HBr with the formation of methyl 3(α)-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate.

Reaction of Methyl 3(α)-Acetoxy-11(β)-bromo-12(α)-hydroxycholanate with Silver Acetate—3.0 gm. of methyl 3(α)-acetoxy-11,12-epoxycholanate were dissolved in 50 ml. of acetone and 8.0 ml. of 1.5 N HBr added. After 15 minutes the solution was neutralized with 11.5 ml. of 0.5 N NaOH. The bromohydrin was extracted with ether, washed with water, and, after drying over Na_2SO_4, the ether was removed under diminished pressure at 30°. The oily residue was thoroughly dried in the desiccator, dissolved in 200 ml. of glacial acetic acid, and shaken mechanically for 15 hours with 1.9 gm. of powdered silver acetate. The solution was decanted into ether and washed with water, dilute HNO_3, 5 per cent $NaHCO_3$ solution, and finally with water. After removal of the ether and crystallization from dilute methanol 1.84 gm. melting at 142–143° were obtained. No depression was found on admixture with methyl 3(α)-acetoxy-11,12-epoxycholanate. When dissolved in acetic acid in the presence of H_2SO_4, the compound showed the typical mutarotation of the epoxide.

The mother liquors yielded a second crop of 550 mg. of crystals, m.p. 81–85°, which on recrystallization from dilute acetone melted unsharply at 83–106°; $[\alpha]_D^{24} = +105°$ (acetic acid). When a trace of concentrated H_2SO_4 was added, the rotation promptly shifted to $[\alpha]_D^{24} = +210°$. 46.2 mg. in 1.0 ml. of absolute ethanol and 1.0 ml. of 1.5 N KOH were boiled under a reflux for 4 hours and a saponification equivalent of 228 was obtained; calculated for $C_{27}H_{42}O_5$ (2 equivalents), 224. Both the optical behavior and the saponification are consistent with methyl 3(α)-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate.

Methyl 3(α)-Acetoxy-12-keto-$\Delta^{9,11}$-cholenate—50 mg. of methyl 3(α)-acetoxy-12-hydroxy-$\Delta^{9,11}$-cholenate were dissolved in 1.0 ml. of pure glacial acetic acid, 0.5 ml. of 1.32 N CrO_3 in acetic acid was added, and the mixture stood at room temperature 20 minutes. Methanol was added and the solution diluted with water. The ether was washed with water, 5 per cent $NaHCO_3$ solution, and again with water. Removal of the ether

yielded 44 mg. of crystals, m.p. 143–145°. After four recrystallizations from methanol it formed rectangular prisms, m.p. 149–150°.

$C_{27}H_{40}O_5$. Calculated, C 72.77, H 9.05; found (J. A.), C 72.31, H 8.64

The product had the characteristic absorption spectrum of an α,β-unsaturated ketone; $\epsilon_{2390} = 11,700$.

Methyl 3(α),11(β)-Diacetoxy-12-ketocholanate—190 mg. of methyl 3-(α),11(β)-diacetoxy-12-hydroxycholanate (amorphous, $[\alpha]_D = +45°$), obtained by the method used in Paper II (1), were dissolved in 3.0 ml. of pure glacial acetic acid, 7.0 ml. of 0.53 N CrO_3 in acetic acid added, and the reaction mixture cooled to between 5–10° for 2 hours. The excess CrO_3 was reduced with methanol, and the solution poured into ether and separated into acidic and neutral fractions in the usual manner. The neutral fraction yielded 68 mg. of crystals from ether-petroleum ether, which after one recrystallization from petroleum ether melted at 109–111°; $[\alpha]_D^{20} = +124°$ (absolute ethanol).

$C_{29}H_{44}O_7$. Calculated.   C 69.02, H 8.83, saponification equivalent 170
            Found (A. M.). " 69.35, " 9.18,   "   " 169

Oxime of Methyl 3(α),11(β)-Diacetoxy-12-ketocholanate—24 mg. of methyl 3(α),11(β)-diacetoxy-12-ketocholanate were heated under a reflux with 35 mg. of hydroxylamine hydrochloride and 68 mg. of sodium acetate trihydrate in 5.0 ml. of ethanol for 5 hours. Upon dilution with water very fine needles were deposited which were twice recrystallized from ethyl acetate-petroleum ether, m.p. 136–138°.

$C_{29}H_{45}O_7N$. Calculated, N 2.71; found,[2] 2.51

No semicarbazone was found either by heating for 5 hours with semicarbazide acetate in pyridine-alcohol solution or by standing at room temperature for several weeks with semicarbazide acetate in pyridine-alcohol solution. Under the latter conditions a small amount of unchanged starting material was recovered but no other crystalline product could be found.

Hydrazone of 3(α)-Hydroxy-11(β)-acetoxy-12-ketocholanhydrazide—200 mg. of methyl 3(α),11(β)-diacetoxy-12-ketocholanate were heated under a reflux for 15 hours with 0.5 ml. of hydrazine hydrate and 1.0 ml. of absolute ethanol. The mixture was cooled in an ice bath; 190 mg. of crystals were obtained which were recrystallized five times from 95 per cent ethanol. The compound was difficult to purify but finally came to a constant

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[2] Microanalysis by Dr. T. S. Ma, Department of Chemistry of the University of Chicago, Chicago.

melting point of 221–223° in a capillary tube. When the melting point was observed under a microscope, the compound melted over 8° in this range. It is noteworthy that, while the prolonged treatment with hydrazine removed the ester groups at C-3 and C-24, the acetoxy group at C-11 was resistant to the reagent. It is possible that epimerization or partial removal of the acetoxy group was responsible for the difficulty in purification.

$C_{26}H_{44}O_4N_4 \cdot H_2O$.   Calculated.   C 63.13, H 9.37, N 11.32
Found (A.M.). " 63.33, " 9.89, " 11.82

3(α),11(β)-Dihydroxy-12-ketocholanic Acid—521 mg. of methyl 3(α),11(β)-diacetoxy-12-ketocholanate were dissolved in 5.0 ml. of 95 per-cent ethanol and 11.7 ml. of 0.6 N KOH in 85 per cent ethanol added. The solution was chilled to 0°. After 3 hours the flask was filled with crystals which after 24 hours at this temperature were filtered, washed with a small amount of cold ethanol, and dried. Yield 414 mg. or 90 per cent of theory. After one recrystallization from water the salt melted at 223–226°; $[\alpha]_D^{25} = +90°$ (0.36 per cent solution in absolute ethanol).

The potassium salt was converted to the acid with acetic acid. This compound proved extremely difficult to crystallize. It forms clumps of soft needles from a concentrated solution in methanol. The best melting point obtained was 147–157°; because of this no analysis was performed. The methyl ester, prepared with diazomethane, proved similarly difficult to crystallize and melted at 189–193°.

Wolff-Kishner Reduction of Hydrazone of 3(α)-Hydroxy-11(β)-acetoxy-12-ketocholanhydrazide—100 mg. of the hydrazide hydrazone were heated at 200° in a sealed tube with 6 ml. of 5 per cent sodium ethylate and 3 drops of hydrazine hydrate for 8 hours. The product obtained on acidification of the reaction mixture was a white amorphous solid weighing 75 mg. As it appeared to be inhomogeneous, it was converted to the methyl ester with diazomethane. The esters were separated by chromatographing on 3 gm. of activated Al_2O_3 according to the scheme given in Table I. Each fraction represented 50 ml. of eluate.

Methyl 3(α)-Hydroxy-Δ11-cholenate—The 24 mg. eluted with benzene-ether were recrystallized from petroleum ether and melted at 96–100°; $[\alpha]_D^{22} = +50°$ (ethyl acetate). Upon admixture with an authentic sample of methyl 3(α)-hydroxy-Δ11-cholenate (m.p. 101–106°) there was no depression of the melting point. It is probable that this product was nevertheless a mixture of methyl lithocholenate and methyl lithocholate similar to that described in Paper IV (5), since these two compounds form mixed crystals.

Methyl 3(α),11(α)-Dihydroxycholanate—19 mg. of the crystalline material eluted with ethyl acetate were recrystallized three times from ethyl acetate-

petroleum ether.   The compound formed clumps of long pointed needles melting at 132–133°; $[\alpha]_D^{22} = +19°$ (ethyl acetate).

$C_{25}H_{42}O_4$.   Calculated, C 73.85, H 10.41; found (A. M.), C 73.67, H 10.56

3(α),11(α)-Dihydroxycholanic Acid—212 mg. of methyl 3(α),11(α)-dihydroxycholanate were hydrolyzed for 1 hour on the steam bath with 5 ml. of 0.5 N alcoholic NaOH.   The solution was poured slowly into dilute H_2SO_4 with stirring, the precipitate filtered, and after one crystallization from ethyl acetate-petroleum ether, melted at from 120–145°.   After three recrystallizations from the same solvent the product sintered to an opaque

TABLE I

Separation of Products from Wolff-Kishner Reduction

Solvent	Ratio	Weight eluted	Description
		mg.	
Benzene-petroleum ether .	1:1	2.4	Oily
" " .	3:1	0.5	"
Benzene		1.0	"
Benzene-ether	9:1	0.5	"
	4:1	14.0	Crystalline
	3:2	10.0	"
	3:2	0.5	Oily
	3:2	0.5	"
	2:3	1.0	"
	1:4	1.3	"
Ether		1.0	"
"		2.8	"
Ethyl acetate .		19.0	Crystalline

mass at 127° and slowly became clear up to 147°; $[\alpha]_D^{25} = +22°$ (absolute ethanol).

$C_{24}H_{40}O_4$.   Calculated, C 73.43, H 10.27; found (J. A.), C 73.98, H 10.62

Methyl 3(α),11(α)-Diacetoxycholanate—46 mg. of methyl 3(α),11(α)-dihydroxycholanate were heated in a sealed tube at 100° for 1 hour with 0.5 ml. of acetic anhydride and 0.5 ml. of pyridine.   The mixture was poured into ice and dilute sulfuric acid, and allowed to stand overnight. The semicrystalline product, recrystallized from dilute methanol, melted at 105–111°.   After two recrystallizations from the same solvent the melting point was 118–119°; $[\alpha]_D^{25} = +13.4°$ (ethyl acetate), $[\alpha]_D^{29} = +7.0°$ (CHCl$_3$).

$C_{28}H_{44}O_6$.   Calculated.   C 70.98, H 9.45, saponification equivalent 163
          Found (A. M.).   " 71.20, " 9.46,   "   "   167

Attempted Dehydration at C-11—Removal of the C-11 hydroxyl group was attempted by the method which Shoppee (6) found to be successful for the cortical substances. 27 mg. of methyl $3(\alpha),11(\alpha)$-dihydroxycholanate in 0.4 ml. of glacial acetic acid and 0.1 ml. of concentrated HCl were heated under a reflux for 30 minutes. The product was esterified with diazomethane and acetylated with acetic anhydride in pyridine. Only methyl $3(\alpha),11(\alpha)$-diacetoxycholanate (21 mg. of crystalline material, m.p. 109–112°) was obtained. This product gave no depression of melting point when mixed with an authentic sample of methyl $3(\alpha),11(\alpha)$-diacetoxycholanate, but, when mixed with methyl $3(\alpha)$-acetoxy-Δ^{11}-cholenate (m.p. 115–117°), the melting point was depressed to 94–97°.

Methyl 3,11-Diketocholanate—25 mg. of slightly impure methyl $3(\alpha),11(\alpha)$-dihydroxycholanate were dissolved in 1.0 ml. of pure glacial acetic acid, 1.0 ml. of 0.6 N CrO_3 in acetic acid added, and the mixture left at room temperature for 20 hours. The acetic acid was removed under diminished pressure at 30° and the residue dissolved in water and ether. The ether solution was washed with dilute sulfuric acid, sodium bicarbonate solution, and water, and then dried over Na_2SO_4. After removal of the ether, the resulting yellow oil was purified by chromatographing over 2 gm. of activated Al_2O_3. 7.0 mg. were eluted with benzene-ether, 4:1, after several small oily fractions had been discarded. This product crystallized in square plates from 60–70° petroleum ether after standing. It melted at 83–85°. Subsequently, when larger amounts were prepared, recrystallization from 60–70° petroleum ether yielded hexagonal plates melting at 84–85°; $[\alpha]_D^{25} = +69°$ (ethyl acetate). Lardon and Reichstein (7) report a melting point of 82–84°; $[\alpha]_D^{17} = +61.7°$ (acetone).

$C_{25}H_{38}O_4$. Calculated, C 74.59, H 9.51; found (J. A.), C 74.40, H 9.30

Monosemicarbazone of Methyl 3,11-Diketocholanate—70 mg. of methyl 3,11-diketocholanate (m.p. 86°) were dissolved in 3.0 ml. of ethanol and 5 ml. of a solution of semicarbazide acetate prepared from 106 mg. of semicarbazide hydrochloride and 126 mg. of sodium acetate trihydrate were added. The mixture stood 16 hours at room temperature and the product was precipitated by the addition of water. Four recrystallizations from methanol yielded rectangular plates melting at 194–197°.

$C_{26}H_{40}O_4N_3$. Calculated, N 9.41; found (J. A.), 9.49

Improved Acetolysis of Methyl 3(α)-Acetoxy-11,12-epoxycholanate—10 gm. of methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate were dissolved in 300 ml. of anhydrous acetic acid and 200 ml. of toluene. 6.0 ml. of 0.96 M H_2SO_4 in acetic acid were added, and the flask immediately chilled to 0° and maintained at this temperature for 16 hours. The solution was poured into

ether, extracted with water and 10 per cent Na_2CO_3 solution, and, after drying over Na_2SO_4, the solvent was removed at 30°.   The residue, weighing 10.3 gm., was dissolved in 50 ml. of pure glacial acetic acid.   100 ml. of 1.0 N CrO_3 in acetic acid were then added and, after 15 minutes at room temperature, the excess CrO_3 was reduced by addition of methanol.   The mixture was poured into a large volume of water and ether and the ether solution washed successively with water, Na_2CO_3 solution, and water. The ether was removed at 30° and the neutral residue (weight 8.4 gm.) in 150 ml. of benzene-petroleum ether, 3:1, was poured over 100 gm. of activated alumina.   The fractions given in Table II were eluted.

Fractions 5 and 6 were combined and after recrystallization yielded 1.70 gm. of methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate, m.p. 107–110°.

TABLE II

Separation of Products from Acetolysis after CrO_3 Oxidation

action No.	Solvent*	Ratio	Weight of eluate	Description
			gm.	
1	Benzene-petroleum ether	3:1	0.399	Oily
2	Benzene		0.987	Crystalline; almost pure
3	Benzene-ether	20:1	2.037	methyl $3(\alpha)$-acetoxy-11,-
4	"	10:1	0.894	12-epoxycholanate
5	"	3:1	1.374	Crystalline
6	"	2:1	0.750	"
7	Ether		0.075	Oily
8	Methanol		1.695	"

* Each fraction represented 1 liter of eluate except Fractions 7 and 8 which were 500 ml.

DISCUSSION

From these experiments, the significant facts emerge that an $11(\beta)$-hydroxyl group does not prevent the formation of ketonic derivatives at C-12 and that the ketone group can be successfully reduced to a methylene without loss of the hydroxyl at C-11.   The failure of the 3,11-dihydroxy-12-ketocholanic acid described by Marker and Lawson to form ketonic derivatives is due to rearrangement by the alkaline hydrolysis involved in its preparation, as will be discussed in Paper VI (8) of this series.

The 3,11-dihydroxycholanic acid isolated by Wolff-Kishner reduction of methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate has been assigned the $11(\alpha)$ configuration on the basis of the ease of acetylation and the relative resistance to dehydration of the C-11 hydroxyl group.   It is interesting to note that whereas the $11(\alpha)$-hydroxyl group is sterically unhindered the ketone group at this position is unreactive as demonstrated by the forma-

tion of a monosemicarbazone from methyl 3,11-diketocholanate. The ketonic group therefore resembles the inert ketonic group at C-11 of the cortical steroids. The cortical steroids must have the hydroxyl group at C-11 in the β configuration, since these substances have been shown to be resistant to acylation and are, moreover, readily dehydrated with mineral acids.

The mechanism of formation of $3(\alpha)',11(\alpha)$-dihydroxycholanic acid by the Wolff-Kishner reduction will be discussed in Paper IV (5).

We wish to express our appreciation of the technical assistance of Miss Joanna Xenos.

SUMMARY

1. Methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate was prepared from methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate by acetolysis and oxidation with CrO_3.

2. This compound readily forms an oxime and a hydrazone.

3. Wolff-Kishner reduction yielded an equal amount of a compound tentatively identified as Δ^{11}-lithocholenic acid and $3(\alpha),11(\alpha)$-dihydroxycholanic acid. The latter product has the C-11 hydroxyl group in the unhindered configuration, since it forms a diacetate under mild conditions and is not readily dehydrated by HCl in acetic acid.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

IV. AN IMPROVED METHOD FOR THE PREPARATION OF 3(α),11(α)-DIHYDROXYCHOLANIC ACID*

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In Paper III of this series (1) a method for the preparation of 3(α),11-(α)-dihydroxycholanic acid from Δ^{11}-lithocholenic acid was reported. Poor yields were obtained, and, since formation of the Δ^{11} acid by a pyrolytic reaction is unsatisfactory, it was desirable to determine whether the 3,11-dihydroxy acid could be prepared directly from desoxycholic acid. It appeared possible that the necessary intermediate, 3(α),11(β)-dihydroxy-12-ketocholanic acid, could be obtained by bromination of methyl 3(α)-acetoxy-12-ketocholanate followed by alkaline hydrolysis of the appropriate 11-bromo-12-keto ester. The bromination of 3(α)-acetoxy-12-ketocholanic acid has been studied by several workers (2–4) who obtained only amorphous products. Recently Seebeck and Reichstein (5) have described two 11-bromo-12-keto esters epimeric at C-11, which were obtained by the bromination procedure of Longwell and Wintersteiner (3) in crystalline form but in poor yield. We were unaware of the preparation of these two epimers by the Swiss investigators and had independently prepared the same compounds.

In attempting to separate a crystalline product from the bromination of methyl 3(α)-acetoxy-12-ketocholanate in acetic acid solution, we noticed that there was an acid fraction in the crude product which suggested that partial hydrolysis had occurred under the influence of the hydrobromic acid formed during the reaction. We therefore carried out the bromination at room temperature and reesterified the reaction product. When a small amount of colored impurity was removed by Al_2O_3, the residue crystallized readily and two epimeric bromo keto esters were separated by fractional crystallization. The 11(α)-bromo ester was converted in excellent yield to 3(α),11(β)-dihydroxy-12-ketocholanic acid, identified as the insoluble sodium or potassium salt. The salt reacts with hydrazine hydrate and the hydrazone can then be reduced to 3(α),11(α)-dihydroxycholanic acid by the Wolff-Kishner method.

<div style="text-align:center">EXPERIMENTAL[1]</div>

Methyl 3(α)-Acetoxy-12 (α)-hydroxycholanate—(Wieland, Dane, and Scholz (6) and Reichstein and Sorkin (7) have described somewhat different methods of preparation.)   400 gm. of methyl desoxycholate were dissolved in 750 ml. of redistilled pyridine, 165 gm. of freshly redistilled acetic anhydride (1.6 moles) added, and the mixture allowed to stand at room temperature for 19 hours.   Ice and water were added and after an interval of 2 to 3 hours the precipitated solid was dissolved in ether.   The pyridine-water-acetic acid mixture was extracted with ether and the combined ether solutions were washed thoroughly with 5 per cent H_2SO_4, 5 per cent Na_2CO_3, and finally with water.   After removal of the ether, the residue was dissolved in methanol and crystallized from this solvent.   Two crops, which together weighed 340 gm., were obtained, m.p. 128–129.5°; $[\alpha]_D^{21}$ = +66° (absolute ethanol).   The yield was 77 per cent of theory.   Very little more crystalline product can be obtained from the mother liquors.

Methyl 3(α)-Acetoxy-12-ketocholanate—463 gm. of methyl 3(α)-acetoxy-12(α)-hydroxycholanate were dissolved in 1500 ml. of glacial acetic acid with gentle warming and the solution cooled to room temperature.   175 gm. of CrO_3 dissolved to 800 ml. of water were added with stirring over 1 to 2 hours.   The reaction mixture was allowed to stand for another hour and 1500 ml. of water were added slowly with continuous stirring.   The crystalline mass was filtered, washed twice by suspension in water, filtered as dry as possible, dissolved in ethanol, and crystallized from this solvent. The product, m.p. 149–151°, weighed 410 gm. and is sufficiently pure for bromination.   The mother liquors yielded 8 gm. with a melting point of 148–150° on concentration.   The yield was 90 per cent of theory.   The pure compound melts at 151–153°; $[\alpha]_D^{23}$ = +111° (absolute ethanol).

Bromination of Methyl 3(α)-Acetoxy-12-ketocholanate—51 gm. of methyl 3(α)-acetoxy-12-ketocholanate were dissolved in 120 ml. of pure glacial acetic acid, 34 ml. of 7.5 N Br_2 (1.1 moles) in acetic acid added, and the flask stoppered and stored in the dark at room temperature for 5 days.   At this time the substitution of bromine was complete.   The acetic acid solution was poured very slowly into a large volume of ice water with vigorous mechanical stirring.   If the addition to the water is made slowly, the product is sandy and easily filtrable; otherwise an oily mass is obtained which is difficult to free from acetic acid.   The precipitate was filtered, washed five times by suspension in water, and dried as thoroughly as possible.   The amorphous bromo ketone was dissolved in dry methanol with gentle warming, 0.5 ml. of concentrated H_2SO_4 added, and the so-

lution allowed to stand at room temperature for 5 hours. The methanol solution was poured into ether, washed with water, dried over Na_2SO_4, and after thorough drying, weighed 57.3 gm. 10 gm. were removed for other purposes and the remainder dissolved in 200 ml. of benzene. In order to study the extent to which the 3-acetoxy group had been hydrolyzed, the benzene solution was divided into two equal portions. One was chromatographed over 60 gm. of Al_2O_3 with 800 ml. of benzene-petroleum ether, 2:1, mixture. 20 gm. of eluate were obtained which failed to crystallize even after inoculation with methyl $3(\alpha)$-acetoxy-$11(\alpha)$-bromo-12-keto-cholanate or with methyl $3(\alpha)$-acetoxy-$11(\beta)$-bromo-12-ketocholanate.

Methyl $3(\alpha)$-Acetoxy-11(α)-bromo-12-ketocholanate—The other portion of the benzene solution (100 ml. = 23.7 gm.) was added to 50 ml. of acetic acid containing 5 ml. of 1.13 M anhydrous $HClO_4$ in acetic acid. The solution was chilled in an ice bath and 30 ml. of acetic anhydride were added.[2] The solution was removed from the ice bath and allowed to stand for 30 minutes. It was then cooled again and the excess acetic anhydride hydrolyzed by the cautious addition of water. The solution was poured into ether and the ether extracted with Na_2CO_3 solution and with water. After removal of the ether, the residue was thoroughly dried and chromatographed over 80 gm. of Al_2O_3 with 700 ml. of benzene-petroleum ether, 1:1. 20 gm. were eluted with this solvent mixture. 3.5 gm. were eluted with 300 ml. of ether-methanol, 4:1. This latter was a dark oil which failed to crystallize. The first eluate was dissolved in methanol and on inoculation with methyl $3(\alpha)$-acetoxy-$11(\alpha)$-bromo-12-ketocholanate crystallized immediately. 15.55 gm. were obtained. The product softened from 124–147° and formed a clear melt at 153°. Three recrystallizations from methanol gave clusters of long prisms with a constant melting point of 163.5–165.5°; $[\alpha]_D^{27} = +44°$ (absolute ethanol).

$C_{27}H_{41}O_5Br$. Calculated, C 61.70, H 7.87, Br 15.21; found, C 61.80, H 7.97, Br 15.44

A mixture with methyl $3(\alpha)$-acetoxy-$11(\beta)$-bromo-12-ketocholanate (8) (m.p. 162–163°) melted at 138–142°.

Methyl $3(\alpha)$-Acetoxy-11(β)-bromo-12-ketocholanate—The mother liquors from the $11(\alpha)$-bromo keto ester upon concentration yielded feathery needles melting unsharply at about 125°. After repeated recrystallization from ethanol and from ethyl acetate-petroleum ether, a compound melting at 160–162° was obtained. This gave no depression when admixed with an authentic specimen of methyl $3(\alpha)$-acetoxy-$11(\beta)$-bromo-12-ketocholanate prepared from the epoxide of Δ^{11}-lithocholenic acid. The yield of this epimer was small.

[2] Schwenk, E., and Whitman, B., personal communication.

Hydrolysis of Methyl 3(α)-Acetoxy-11(α)-bromo-12-ketocholanate—200 mg. samples of the α-bromo keto ester were dissolved in 15 ml. of ethanol and 5.0 ml. of 2.0 N aqueous KOH added. The solutions were stored at room temperature (between 27–30°) and titrated with 0.1 N H_2SO_4 at intervals. After 6, $21\frac{1}{2}$, and 48 hours, 76.8, 92.5, and 94 per cent of the calculated amount of base (3 equivalents) were consumed. 17.09 gm. of 11(α)-bromo keto ester were dissolved in 850 ml. of ethanol, 280 ml. of 2.0 N KOH were added, and the mixture stood for 48 hours at room temperature (28–30°). The alkaline solution was chilled and acidified under ether. The ether extract was washed with water and dried over Na_2SO_4 and the ether distilled. The residue was dissolved in 100 ml. of absolute ethanol and chilled to 0°, and 6.0 ml. of 5.0 N aqueous KOH added. The insoluble potassium salt was filtered and washed with ice-cold water. It weighed 10.7 gm. or 75 per cent of theory. The potassium salt melted at 217–220° after one recrystallization from water; $[\alpha]_D^{24} = +90°$ (0.36 per cent in absolute ethanol). The sodium salt prepared in similar fashion after two recrystallizations from dilute ethanol melted at 195–197°; $[\alpha]_D^{26} = +98°$ (0.50 per cent in absolute ethanol).

Hydrazone of 3(α),11(β)-Dihydroxy-12-ketocholanhydrazide—Since the hydrazone of the sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid was unsatisfactory for analysis, the hydrazone of the hydrazide was prepared. Methyl 3(α),11(β)-dihydroxy-12-ketocholanate was heated with excess hydrazine hydrate in absolute ethanol for 1 hour. The reaction product crystallized from the hot solution and was recrystallized from ethanol. Fine needles melting at 229–232° were obtained, which similarly gave an unsatisfactory analysis.

$C_{24}H_{42}O_3N_4 \cdot H_2O$. Calculated, N 12.38; found, 11.97

Wolff-Kishner Reduction of 3(α),11(β)-Dihydroxy-12-ketocholanic Acid— 25 gm. of the sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid were dissolved in 350 ml. of absolute ethanol under a reflux and 20 ml. of hydrazine hydrate added. Heating was continued for 30 minutes and then about half the alcohol was removed by distillation. A heavy mass of crystals formed which were removed by filtration and combined with a second crop obtained by further concentration of the mother liquors. Weight, 20.8 gm. A third crop weighed 1.36 gm. The mother liquors were not further investigated. The yield was 87 per cent of theory. The compound is extremely hygroscopic and gave an unsatisfactory analysis. The hydrazone was reduced with sodium ethylate at 200° in a sealed tube in the presence of hydrazine hydrate. The products proved to be a similar mixture to that described in Paper III (1) and were separated in the following manner.

The contents of the bomb tube were rinsed into a flask and neutralized with HCl, and the concentration of alcohol and water adjusted so that, for each 4 gm. of hydrazone reduced, the product was dissolved in 100 ml. of hot ethanol and 200 ml. of water. This solution was heated to boiling and 100 ml. of hot 0.3 N Ba(OH)$_2$ were added with stirring. The flask was stoppered and allowed to stand overnight. The insoluble barium salt was filtered and dried. In several experiments this weighed about 50 to 55 per cent of the weight of the hydrazone which had been reduced.

Methyl 3(α),11(α)-Dihydroxycholanate—The filtrate from the barium salt was acidified and the acid isolated by extraction with ether in the usual manner. This was dried and esterified with methanol and concentrated H$_2$SO$_4$ at room temperature. The ester was crystallized from benzene-petroleum ether and a crop of large prisms formed which melted at 125–127° and gave no depression with an authentic specimen of methyl 3(α),-11(α)-dihydroxycholanate. The yield obtained in several experiments corresponded to from 20 to 30 per cent of theory. The product is slightly impure and is most conveniently purified as the methyl ester diacetate.

Methyl 3(α),11(α),12(β)-Triacetoxycholanate (9)—The mother liquors from methyl 3(α),11(α)-dihydroxycholanate either fail to yield further crystalline material or deposit oily crystals difficult to separate. Several batches of these mother liquors were combined (weight, 34.0 gm.) and acetylated with acetic anhydride and HClO$_4$ as catalyst. Crystallization from methanol yielded 1.4 gm. of a product melting at 160–173°. Recrystallization from ethyl acetate gave 800 mg. of a compound melting at 187–189°, which was identical with methyl 3(α),11(α),12(β)-triacetoxy-cholanate described in Paper VI (9). In the further purification of the oily residues 1.2 gm. of the same product were obtained.

Methyl 3(α),11(α)-Diacetoxycholanate—The oily esters after crystallization of methyl 3(α),11(α),12(β)-triacetoxycholanate were chromatographed several times on aluminum oxide. From the early eluates 1.17 gm. of methyl acetoxylithocholate and 600 mg. of methyl acetoxy-Δ^{11}-lithocholenate (identified by melting point and titration with bromine) were isolated. The major proportion of the material (20.3 gm.) was eluted with petroleum ether, petroleum ether-benzene mixtures, and benzene. From these fractions 10.16 gm. of methyl 3(α),11(α)-diacetoxy-cholanate (m.p. 116–118°) were obtained.

Methyl 3(α),11(α),12(α)-Triacetoxycholanate (9)—0.75 gm. of this product, m.p. 163–165°, was isolated from the benzene-ether, 1:3, eluates. Its constitution is discussed in Paper VI (9).

No other crystalline products were isolated from the soluble barium salts.

Methyl 3(α)-Acetoxycholanate and Methyl 3(α)-Acetoxy-Δ^{11}-cholenate—

22.9 gm. of insoluble barium salts were acidified and extracted with ether. The acid fraction was esterified with methanol and concentrated H_2SO_4 at room temperature.   The methyl esters were acetylated with acetic anhydride and $HClO_4$ as a catalyst and, upon crystallization from acetone, 16.2 gm. of a product melting from 118–124° were obtained.   This was separated by the procedure of Seebeck and Reichstein (5).   It was dissolved in $CHCl_3$ and allowed to stand 24 hours at room temperature with

TABLE I

Separation of Methyl Lithocholate and Methyl 3(α)-Acetoxy-11,12-epoxycholanate

Fraction No.	Solvent*		Ratio	Weight eluted	Description
				gm.	
1	Benzene-petroleum ether		1:19	4.16	Methyl 3(α)-acetoxycho-
2	"	"	1:19	0.49	lanate, m.p. 134.5–135.5°,
					$[α]_D = +47.6°$ (acetone)
3	"	"	1:19	0.001	Not investigated
4	"	"	1:9	0.003	" "
5	"	"	1:9	0.005	" "
6	"	"	1:4	0.031	" "
7	"	"	1:4	0.026	" "
8	Benzene	.		0.033	" "
9	"			0.052	" "
10	Benzene-ether		4:1	1.13	Methyl 3(α)-acetoxy-11,12-
11	"		4:1	0.34	epoxycholanate, m.p. 137–
12	"		4:1	0.19	140°, $[α]_D = +33°$ (toluene)
13	"		4:1	0.10	$[α]_D = +60°$ (acetone)
14	"		3:1	0.10	Non-crystalline
15	"		1:3	0.26	"
16	"		1:3	0.18	"
17	Ether			0.15	"
18	"			0.03	"

* All fractions were 500 ml. except Fraction 1 which was 1000 ml.

400 ml. of 0.265 N perbenzoic acid in $CHCl_3$.   Titration of aliquots showed that the mixture had reacted with 3.22 gm. of perbenzoic acid or 60 per cent of theory if the material were methyl 3(α)-acetoxy-$Δ^{11}$-cholenate.

Upon standing for an additional 24 hours, no further uptake of perbenzoic acid occurred.   The $CHCl_3$ solution was washed with dilute Na_2CO_3 solution and with water and distilled to dryness.   The product was dried in a vacuum and dissolved in 500 ml. of benzene-petroleum ether mixture, 1:19.   250 ml. of this solution (8.04 gm.) were poured over

a column of 85 gm. of Al_2O_3 and the mixture separated according to the scheme shown in Table I.

DISCUSSION

The purpose of this investigation was to devise a method for the production of 3,11-dihydroxycholanic acid by a simple procedure. This has been accomplished. The configuration of the product and the structure of the other compounds formed are significant in the interpretation of the mechanism and it is this phase of the investigation which we propose to discuss.

In Paper II of this series (8) it was concluded that fission of the epoxide, methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate, by organic or mineral acids led to the introduction of the anionic constitutent in the β configuration at C-11, while the proton reacted with the oxygen atom after rupture of the epoxide ring to form a hydroxyl group having the α configuration at C-12. In the same investigation, methyl $3(\alpha)$-acetoxy-11(β)-bromo-12-ketocholanate was prepared. If the configuration of this latter substance has been correctly assigned, it is clear that the diastereoisomer must have the 11(α) configuration. Consideration of the properties of the two epimers as discussed below indicates that the structures are correctly assigned and provides further evidence for the validity of the conclusions previously drawn regarding the opening of the epoxide ring and the configuration of the C-12 hydroxyl group of desoxycholic acid.

An inspection of molecular models demonstrates that a substituent at carbon atom 11 should be markedly hindered in one configuration and relatively unhindered in the alternative steric position. The hindered configuration is designated β according to the generally accepted system of nomenclature and is understood to mean a substituent cis with respect to the angle methyl groups C-18 and C-19. It could be anticipated, therefore, that the bromination of a 12-keto bile acid would lead to the formation of a hindered isomer in small amounts. This is in accord with the findings reported here and with the results of Seebeck and Reichstein (5) who also obtained very small yields of this isomer.

The results obtained when the two epimeric bromo keto esters are hydrolyzed with aqueous base are similarly consistent and in harmony with the modern conception of a displacement reaction involving Walden inversion (10). When the 11(β)-bromo-12-keto ester, in which the halogen is in the hindered configuration, is hydrolyzed by aqueous alcoholic base at room temperature, the entering hydroxyl group can readily approach the molecule at the sterically unhindered face of carbon atom 11. Under these circumstances the halogen is then displaced and the hydroxyl group attaches to C-11 with inversion of configuration so that the product should

be $3(\alpha),11(\alpha)$-dihydroxy-12-ketocholanic acid. The reaction with base was relatively rapid with this epimer,[3] since 3 equivalents were consumed in less than 3 hours at room temperature.   In contrast to this result, when the $11(\alpha)$-bromo-12-keto ester was hydrolyzed with the same concentration of alkali, the reaction was much slower and was incomplete even after 20 hours.   Since the displacement of halogen from this epimer takes place by the approach of the hydroxyl ion from the hindered side of carbon atom 11, this replacement should proceed more slowly.

It has been assumed in the preceding discussion that an inversion of configuration has occurred.   This can be demonstrated with a considerable degree of certainty and without recourse to an investigation of the kinetics of the reaction.   The product formed by acetolysis of methyl $3(\alpha)$-acetoxy-11,12-epoxycholanate is methyl $3(\alpha),11(\beta)$-diacetoxy-12(α)-hydroxycholanate not only by analogy to the reaction of the epoxide with hydrobromic acid but also because the 11-acetoxy group exhibits such marked resistance to hydrolysis with base.   When the hydroxyl group at C-12 is oxidized to a ketone, the product methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate is readily hydrolyzed by alkali at room temperature.   Since the saponification of an acetoxy group is unlikely to effect a change in configuration, the product of hydrolysis is $3(\alpha),11(\beta)$-dihydroxy-12-ketocholanic acid.   This substance forms an insoluble sodium or potassium salt and is thus readily identified.   This same insoluble salt is formed when methyl $3(\alpha)$-acetoxy-$11(\alpha)$-bromo-12-ketocholanate is hydrolyzed by base at room temperature, so that the replacement of halogen by hydroxyl in this diastereoisomer is accomplished with Walden inversion.   Since the $11(\beta)$-bromo ester is hydrolyzed under the same conditions to a different dihydroxy keto acid (11) it is clear that inversion had occurred with both epimers.

The two diastereoisomeric $3(\alpha),11$-dihydroxy-12-ketocholanic acids similarly react in accordance with the configurations which have been assigned. $3(\alpha),11(\alpha)$-Dihydroxy-12-ketocholanic acid forms a diacetoxy methyl ester which is described in Paper V (11).   In two experiments we attempted unsuccessfully to prepare a diacetate of methyl $3(\alpha),11(\beta)$-dihydroxy-12-ketocholanate.   While no emphasis need be given to the negative result, it is important that the $11(\alpha)$-hydroxyl group is *not* subject to steric hindrance.   This has previously been demonstrated by the formation of a diacetate from the methyl ester of $3(\alpha),11(\alpha)$-dihydroxycholanic acid.   It is interesting to note that whereas the $11(\alpha)$-hydroxyl is sterically unhindered and forms derivatives readily, the ketone group at this position is unreactive as has previously been noted for the cortical steroids.

In Paper III (1) of this series the 3,11-dihydroxycholanic acid obtained

[3] Gallagher, T. F., and Long, W. P., unpublished observations. See also Paper V of this series (11).

from the Wolff-Kishner reduction of methyl $3(\alpha),11(\beta)$-diacetoxy-12-ketocholanate was assigned α configuration at C-11 because of the ease of acetylation and the resistance of this hydroxyl to dehydration by hydrochloric acid. The same product was obtained in this investigation from the reduction of the unesterified hydrazone. It is necessary therefore to account not only for the inversion of the hydroxyl group at C-11 but for the other products formed during reduction of the 12-ketone. It can be assumed that the hydrazone of $3(\alpha),11(\beta)$-dihydroxy-12-ketocholanic acid (I) is in equilibrium with the $11(\alpha)$ epimer (II) in the presence of base,

before reduction of the hydrazone to a methylene group. The equilibrium product undergoes reduction with the formation of two $3(\alpha),11$-dihydroxycholanic acids (III and IV) epimeric at C-11.

The formation of $3(\alpha),11(\alpha)$-dihydroxycholanic acid from the reduction of an $11(\beta)$-hydroxy-12-keto acid is understandable if an equilibrium of the hydrazones as outlined in the accompanying formulas is postulated. It is uncertain from our results whether $3(\alpha),11(\beta)$-dihydroxycholanic acid (IV) is formed only as an intermediate or whether it is present in the final reaction mixture. We were unable to isolate the product but no emphasis should be placed upon this fact, since the products are difficult

to separate after the initial crystallization of methyl-3(α),11(α)-dihydroxy-cholanic acid. It is certain, however, that if present, the 11(β) epimer constitutes a relatively minor fraction of the total product.

We have shown that the 11(α) epimer is stable under the conditions of the reaction by comparing the yield of this product when the bomb tube was heated for 2 hours and for 8 hours at 200° with the same concentrations of sodium ethylate. No difference in the yield of either 3(α),11(α)-dihydroxycholanic acid or the mixture of lithocholic (VI) and lithocholenic (V) acids was found, within the limits of accuracy imposed by the technique of isolation; it is improbable therefore that any considerable amount of either lithocholic or lithocholenic acid resulted from dehydration of 3(α),-11(α)-dihydroxycholanic acid after formation of this product. It is possible, however, that the lithocholenic acid results from dehydration of a labile 11(β)-hydroxyl group under the influence of the hot sodium ethylate. This loss of water must occur after reduction of the hydrazone to a methylene group, since otherwise $\Delta^{9,11}$-lithocholenic acid should be the principal reaction product, provided there is no shift of the double bond. This latter possibility cannot readily be excluded, since Seebeck and Reichstein (5) obtained Δ^{11}-lithocholenic acid as well as $\Delta^{9,11}$-lithocholenic acid from the Wolff-Kishner reduction of 3(α)-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid even when the purest preparations were reduced. It is significant that $\Delta^{9,11}$-lithocholenic acid was either absent or present in small traces, as judged by the chromatogram of the oxides, following Wolff-Kishner reduction of 3(α),11(β)-dihydroxy-12-ketocholanic acid. It is probable nevertheless that in the mixture obtained by us there were present other unsaturated acids such as $\Delta^{8,9}$ or $\Delta^{8,11}$, since the amount of perbenzoic acid consumed was greater than could be accounted for by the quantity of methyl 3(α)-acetoxy-11,12-epoxycholanate isolated. Furthermore the amount of perbenzoic acid consumed was in excess of 1 mole when allowance is made for the lithocholic acid obtained (57 per cent) and this finding is consistent with the possibility that $\Delta^{8,9}$ or $\Delta^{8,14}$ acids were present in the mixture, since these substances form dienic acids when treated with perbenzoic acid (12, 13).

A portion of the lithocholic acid in the reaction product could be formed from unchanged 3(α)-hydroxy-12-ketocholanic acid present as a contaminant in the bromination product and adsorbed on or precipitated with the insoluble sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid. However, even when carefully purified preparations of methyl 3(α),-11(β)-diacetoxy-12-ketocholanate were subjected to the Wolff-Kishner reaction, a smaller but significant amount of lithocholic acid was formed. It is possible that a certain amount of osazone is formed in the reaction of the ketol with hydrazine and that this product was directly reduced to

lithocholic acid. This explanation is objectionable, since derivatives of C-11 ketone groups are formed with difficulty although the activating influence of the hydrazone at C-12 may be of importance and effect the formation of an osazone to a limited extent. It is also possible that some lithocholic acid results from the reduction of Δ^{11}-lithocholenic acid by the products of alkaline decomposition of the excess hydrazine present in the reaction mixture. On the basis of present evidence, it is impossible to decide the mechanism by which both oxygen atoms in Ring C are lost with the formation of a saturated derivative.

It is likewise certain that Wolff-Kishner reduction of the ketol structure in Ring C leads to the formation of 3,11,12-trihydroxy acids. These may be the result of an abnormal course of the Wolff-Kishner reaction of the type investigated by Dutcher and Wintersteiner (14). These authors, however, found that the reduction of a semicarbazone to a secondary alcohol appeared to be limited to C-3 in the steroid nucleus and could be prevented completely if the reduction were carried out in the presence of excess hydrazine. Since in our experiments the reduction was invariably conducted with excess hydrazine present, this explanation appears to be unlikely. It is, however, possible that conversion of the 11-hydroxy-12-keto bile acid to the hydrazone is incomplete, since, in the course of heating the alcoholic solution of the sodium salt with hydrazine hydrate, a portion of the product may have been converted into the acid of Marker and Lawson (2). It is known (15) that this latter compound can form 3,11,12-trihydroxy acids under the conditions of the Wolff-Kishner reaction and this fact is confirmed by investigations described in Paper VI of this series (9). Since $3(\alpha),11(\beta)$-dihydroxy-12-ketocholanic acid can be converted to the acid of Marker and Lawson by heating with base, it is reasonable to assume that at least a portion of the sodium salt of $3(\alpha),11(\beta)$-dihydroxy-12-ketocholanic acid was so transformed in the manipulation of the compound before the hydrazone had been formed. This explanation would also account for the difficulty encountered in obtaining analytically pure samples of the hydrazone. If this hypothesis is correct, a significant improvement in the process could be achieved by preparation of the hydrazone under less drastic conditions.

We wish to express our appreciation of the technical assistance of Miss Joanna Xenos.

SUMMARY

1. A procedure for the conversion of desoxycholic acid to 3,11-dihydroxycholanic acid has been described.

2. The process involves the bromination of methyl $3(\alpha)$-acetoxy-12-

ketocholanate, hydrolysis of the mixture at room temperature with NaOH, and isolation of 3(α),11(β)-dihydroxy-12-ketocholanic acid as the insoluble sodium salt. The latter substance forms a hydrazone which can be reduced by the Wolff-Kishner method to a mixture of which 3(α),11(α)-dihydroxycholanic acid, lithocholic acid, Δ^{11}-lithocholenic acid, and 3(α),-11,12-trihydroxycholanic acids are the principal constituents.

3. The configuration of substituents at C-11 of the steroid nucleus has been discussed.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

V. PREPARATION OF 3(α),11(α)-DIHYDROXY-12-KETOCHOLANIC ACID AND THE PRODUCTS OBTAINED BY WOLFF-KISHNER REDUCTION[*]

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The introduction of an oxygen function at C-11 of the steroid nucleus as the first stage in the partial synthesis of adrenal cortical hormones has been the subject of investigation in this and other laboratories for some time. It had been shown that 3(α),11(β)-dihydroxy-12-ketocholanic acid could be successfully reduced to a 3,11-dihydroxycholanic acid (1, 2) by the Wolff-Kishner method and it was therefore desirable to determine whether the diastereoisomer, 3(α),11(α)-dihydroxy-12-ketocholanic acid, would behave in similar fashion. The latter substance was prepared by low temperature alkaline hydrolysis of methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate (1) in crystalline form and in good yield. The product proved capable of forming ketonic derivatives and upon reduction by the Wolff-Kishner method formed 3(α),11(α)-dihydroxycholanic acid and Δ[11]-lithocholenic acid as the principal products. The reaction was therefore similar to the reduction of 3(α),11(β)-dihydroxy-12-ketocholanic acid previously studied by Gallagher and Long (1).

Neither the 3(α),11(α)-dihydroxy-12-ketocholanic acid described in this report nor the 3(α),11(β)-dihydroxy-12-ketocholanic acid described by Long and Gallagher (2) is identical with the compound prepared by Marker and Lawson (3) and formulated by these authors as 3(α),11-dihydroxy-12-ketocholanic acid. It is apparent that the structure of the latter product must be revised; this problem is dealt with in Paper VI (4).

EXPERIMENTAL[1]

3(α),11(α)-Dihydroxy-12-ketocholanic Acid—1.004 gm. of methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate, obtained from methyl 3(α)-ace-

[1] All melting points are corrected. The microanalyses were performed by Mr. John De Lucia (J. De L.), New York, and Professor A. J. Haagen-Smit (A. J. H.-S.), California Institute of Technology. We wish to express our appreciation for this service.

toxy-11,12-epoxycholanate by treatment with hydrobromic acid and subsequent oxidation with CrO_3, were dissolved in 100 ml. of ethanol, 25.0 ml. of 4.2 N NaOH were added, and the solution was allowed to stand at room temperature for 3 hours. The product was acidified and extracted thoroughly with ether. The ether solution was washed with water, dried with Na_2SO_4, and evaporated to dryness. The product after two recrystallizations from ethyl acetate melted at 197–199°; $[\alpha]_D^{25} = +65.6°$ (absolute ethanol). A mixture with an authentic specimen of the Marker and Lawson acid (m.p. 205°, obtained by vigorous alkaline hydrolysis of methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate) (5) melted at 177°.

$C_{24}H_{38}O_5$. Calculated, C 70.90, H 9.42; found (J. De L.), C 70.92, H 9.42

Oxime of 3(α),11(α)-Dihydroxy-12-ketocholanic Acid—226 mg. of 3(α),-11(α)-dihydroxy-12-ketocholanic acid were heated under a reflux with 500 mg. of hydroxylamine hydrochloride and 1 gm. of sodium acetate trihydrate in 10 ml. of ethanol for $2\frac{1}{2}$ hours. The reaction mixture was diluted with water and extracted with ethyl acetate. The ethyl acetate was washed with water and evaporated to dryness. The product crystallized from ethyl acetate-petroleum ether as fine needles and after three recrystallizations melted at 185–188° with decomposition.

$C_{24}H_{39}O_5N$. Calculated. C 68.37, H 9.32, N 3.32
Found (J. De L.). " 68.25, " 9.29, " 3.24

Methyl 3(α),11(α)-Diacetoxy-12-ketocholanate—621 mg. of 3(α),11(α)-dihydroxy-12-ketocholanic acid were dissolved in 10 ml. of methanol and esterified with diazomethane. As the ester failed to crystallize, the oily product was dissolved in 5 ml. of glacial acetic acid and acetylated[2] by the addition of 5 ml. of acetic anhydride in the presence of 0.5 ml. of 70 per cent $HClO_4$. The solution was chilled in an ice bath and diluted with water, and the ester was extracted with ether. Upon recrystallization from acetone-90–100° petroleum ether the compound formed long, silky needles. After two recrystallizations from the same solvent the product melted at 153–154°; $[\alpha]_D^{25} = +38°$ (absolute ethanol).

$C_{29}H_{44}O_7$. Calculated, C 69.02, H 8.79; found (J. De L.), C 68.91, H 8.81

Wolff-Kishner Reduction of 3(α),11(α)-Dihydroxy-12-ketocholanic Acid— 6.880 gm. of 3(α),11(α)-dihydroxy-12-ketocholanic acid were dissolved in 5 ml. of absolute ethanol and heated under a reflux for 3 hours with 1.0 ml. of hydrazine hydrate. The alcohol was partially removed under diminished pressure, and the residue dried and transferred to a glass tube with 5.0 ml. of absolute ethanol. 25.0 ml. of sodium ethylate, prepared from 2.36 gm. of

sodium, and 0.25 ml. of hydrazine hydrate were added, and the glass tube inserted in a steel bomb and heated at 200° for 90 minutes. The reaction mixture was neutralized and the product separated into soluble and insoluble barium salts by the procedure of Gallagher and Long (1).

Methyl 3(α),11(α)-Dihydroxycholanate—The soluble barium salt was acidified and extracted with ether. 3.698 gm. of acids were obtained, which were esterified with diazomethane and crystallized from ethyl acetate-petroleum ether. 1.077 gm. melting at 93–116° were obtained, which upon recrystallization from the same solvents melted at 133–134°; $[\alpha]_D = +21.7°$ (95 per cent ethanol). There was no depression of the melting point upon admixture with an authentic specimen of methyl 3(α),11(α)-dihydroxycholanate.

$C_{25}H_{42}O_4$. Calculated, C 73.84, H 10.41; found (J. De L.), C 73.53, H 10.73

The product was further characterized by conversion to the diacetate which melted at 116–118° and gave no depression when mixed with an authentic sample of methyl 3(α),11(α)-diacetoxycholanate.

The non-crystalline residue (2.62 gm.) was acetylated and chromatographed twice on Al_2O_3. 1.413 gm. of crystalline product melting at 112–116° were obtained, which showed no depression of melting point when admixed with an authentic specimen of methyl 3(α),11(α)-diacetoxycholanate. The total yield of 3(α),11(α)-dihydroxycholanic acid corresponds to 32.6 per cent of theory.

For the purpose of estimating the amount of 3,11,12-trihydroxycholanic acids present in the mixture, advantage was taken of the specific action of lead tetraacetate upon vicinal hydroxyl groups. The dialdehyde produced from the oxidation of the 11,12-glycol was then further oxidized with CrO_3 after protection of the intact hydroxyl at C-3 by acetylation and the amount of acidic product taken as a rough measure of the 3,11,12-trihydroxycholanic acid present in the mixture. The non-crystalline residues from the methyl 3(α),11(α)-diacetoxycholanate were combined, saponified completely, and the acid converted to the methyl ester. The oily product was dissolved in glacial acetic acid and oxidized with lead tetraacetate at room temperature. The reaction consumed approximately 3 equivalents after 72 hours. The reaction product was extracted with ether, dried, and acetylated with acetic anhydride with 0.2 ml. of $HClO_4$ as a catalyst. The acetylated product was oxidized with CrO_3 in glacial acetic acid overnight at room temperature. The neutral fraction was then isolated by extraction with ether and washing with dilute base and with water.

The amorphous neutral residue obtained from 1.15 gm. of acetylated oxidation product weighed 463 mg. and was chromatographed on alumina. 73 mg. of methyl 3(α),11(α)-diacetoxycholanate (m.p. 114–116°, no depres-

sion on admixture with an authentic sample) were obtained. The remainder of the material was oily and could not be crystallized. A control experiment in which the acetylated product was oxidized with CrO_3 without previous oxidation with lead tetraacetate gave an almost quantitative yield of neutral material. These results indicate that about 15 per cent of the soluble barium salt was 3,11,12-trihydroxycholanic acid.

Methyl 3(α)-Acetoxy-Δ¹¹-cholenate and Methyl 3(α)-Acetoxycholanate—The insoluble barium salt weighed 2.42 gm. or 32 per cent of theory calculated as the salt of lithocholenic acid. This was acidified, and the acids isolated by extraction with ether were esterified with diazomethane. Acetylation with acetic anhydride and pyridine yielded 2.15 gm. of crystalline product which was separated by the procedure of Seebeck and Reichstein (6). It was dissolved in chloroform and treated with a considerable molar excess of

TABLE I

Separation of Products from Insoluble Barium Salts

Fraction No. (200 ml. each)	Solvent		Ratio	Weight of eluate	Description
				mg.	
	Petroleum ether			115	Crystalline
		zene	5:1	180	"
			1:1	183	"
	Benzene			68	Oily
	Benzene-ether		5:1	45	
	Ether			44	

perbenzoic acid for 16 hours at room temperature. The consumption of perbenzoic acid was almost exactly the theoretical value calculated for methyl lithocholenate. 2.2 gm. of product were obtained. 220 mg. were set aside and the remainder, upon crystallization from acetone and from petroleum ether, yielded 850 mg. of material melting at 144–145°; $[α]_D$ = +56.4° (acetone), +34.0° (toluene). This showed no depression of melting point on admixture with methyl 3(α)-acetoxy-11,12-epoxycholanate. The mother liquors weighed 748 mg. and were chromatographed on 20 gm. of alumina. The fractions shown in Table I were obtained.

Fraction 1 after four recrystallizations from petroleum ether gave 18 mg. of needles, m.p. 130–131°; $[α]_D$ = +47.4° (in 95 per cent ethanol). No depression of the melting point was observed on admixture with methyl 3(α)-acetoxycholanate.

$C_{27}H_{44}O_3$. Calculated, C 74.95, H 10.25; found (A. J. H.-S.), C 75.60, H 10.24

Fractions 2 and 3 were combined and after two recrystallizations from petroleum ether 60 mg. of product melting at 142–144°, $[\alpha]_D = +52.2°$ (acetic acid), were obtained. This compound did not depress the melting point of an authentic sample of methyl 3(α)-acetoxy-11,12-epoxycholanate upon admixture. Fractions 4, 5, and 6 proved difficult to purify and were discarded.

The mother liquors from the recrystallization of Fractions 1, 2, and 3 (270 mg.) were combined and again chromatographed on alumina. Despite careful search and three additional chromatographic separations no substance other than small amounts of methyl 3(α)-acetoxycholanate and methyl 3(α)-acetoxy-11,12-epoxycholanate were obtained.

DISCUSSION

From these experiments and the work of Gallagher and Long (1) it would appear that the configuration of the 11-hydroxyl group in an 11-hydroxy-12-ketosteroid is without influence on the course of the reduction of the carbonyl group to a methylene group by the Wolff-Kishner method. The same products, i.e. 3(α),11(α)-dihydroxycholanic acid, 3(α)-hydroxy-Δ^{11}-cholenic acid, small amounts of lithocholic acid, and 3,11,12-trihydroxy-cholanic acids, are obtained from both the 11(α)- and 11(β)-hydroxy-12-ketocholanic acids. These results substantiate the view that in the course of the Wolff-Kishner reduction at least partial inversion at C-11 invariably occurs in that both diastereoisomers yield the same products. Subsequent to the reduction of the 12-ketonic group, the hydroxyl in the β configuration at C-11 is eliminated with the introduction of a double bond from C-11 to C-12, whereas the 11(α)-hydroxyl group is stable under the experimental conditions. The small amounts of lithocholic acid consistently obtained in the reaction products may be due to reduction of the Δ^{11}-lithocholenic acid by the products of alkaline decomposition of hydrazine.

The findings recorded here would appear to make unnecessary the separation of the insoluble sodium salt of 3(α),11(β)-dihydroxycholanic acid in the procedure of Gallagher and Long (1) for the introduction of the 11-hydroxyl group in the steroid nucleus, since both epimers at C-11 yield the same products. It may be found, however, that despite the slightly higher yield of 3(α),11(α)-dihydroxycholanic acid from 3(α),11(α)-dihydroxy-12-ketocholanic acid the accumulation of side products from the bromination of the 12-ketone may make the separation of the desired product more difficult.

It is likewise apparent from these experiments that 3(α),11(α)-dihydroxy-12-ketocholanic acid is in part converted to 3,11,12-trihydroxy-cholanic acids in the Wolff-Kishner reduction, since there was a noticeable

reaction with lead tetraacetate in the products remaining after isolation of the major portion of $3(\alpha), 11(\alpha)$-dihydroxycholanic acid. These trihydroxy acids are consistently found as products of the Wolff-Kishner reduction of both diastereoisomeric $3(\alpha), 11$-dihydroxy-12-ketocholanic acids despite the presence of excess hydrazine in the reaction mixture. Their formation has been discussed in Paper IV (1) of this series. Since the principal object of this investigation was the configuration of the 3,11-dihydroxy-cholanic acid formed in the Wolff-Kishner reaction, these side products were not further investigated.

SUMMARY

1. $3(\alpha), 11(\alpha)$-Dihydroxycholanic acid was obtained by Wolff-Kishner reduction of $3(\alpha), 11(\alpha)$-dihydroxy-12-ketocholanic acid.

2. Lithocholic and Δ^{11}-lithocholenic acids were likewise isolated from the products of reaction.

3. The presence of 3,11,12-trihydroxycholanic acids in the reaction products was indicated by oxidation with lead tetraacetate.

4. The Wolff-Kishner reduction of $3(\alpha), 11(\alpha)$-dihydroxy-12-ketocholanic acid yields qualitatively the same products as that of the $11(\beta)$ diastereoisomer.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

VI. THE STRUCTURE OF THE "3(α),11-DIHYDROXY-12-KETOCHOLANIC ACID" OF MARKER AND LAWSON AND OF THE PRODUCTS OBTAINED BY "WOLFF-KISHNER REDUCTION"*

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In 1938, Marker and Lawson (1) prepared a compound to which they assigned the structure 3(α),11-dihydroxy-12-ketocholanic acid. Longwell and Wintersteiner (2) in 1940 found that this compound did not give the expected ketonic derivatives. Upon treatment with hydrazine and sodium ethylate at 200° the compound yielded a product melting at 162–163° with the empirical formula $C_{24}H_{38}O_3 \cdot \frac{1}{2}H_2O$. This was regarded as resulting from the elimination of both oxygen atoms from Ring C. Subsequently, Marker, Shabica, Jones, Crooks, and Wittbecker (3) reinvestigated the reaction of the compound with hydrazine and sodium ethylate and isolated a substance melting at 136° with the empirical formula $C_{24}H_{40}O_5$. This product was assigned the structure 3,11,12-trihydroxycholanic acid and its formation was interpreted as involving an abnormal Wolff-Kishner reduction of the type studied by Dutcher and Wintersteiner (4).

Long and Gallagher (5) prepared 3(α),11(β)-dihydroxy-12-ketocholanic acid, which differed from the Marker and Lawson acid in its physical properties and, in addition, readily formed ketonic derivatives. Later Gallagher and Hollander (6) described the epimer of this compound, *viz.* 3(α),11(α)-dihydroxy-12-ketocholanic acid, which likewise differed from the acid of Marker and Lawson both in its physical properties and in its ability to form ketonic derivatives. A comparison of the acetylated methyl esters of these two 3(α),11-dihydroxy-12-ketocholanic acids epimeric at C-11 with the corresponding derivative of the Marker and Lawson acid is presented in Table I. From these data the conclusion can be drawn that the structure originally assigned to their compound by Marker and Lawson was incorrect.

In view of the following facts, it seems probable that the Marker and Lawson acid has the carbonyl group at C-11 rather than C-12: (1) Both 3(α),11-dihydroxy-12-ketocholanic acids epimeric at C-11 readily form

ketonic derivatives, whereas the compound of Marker and Lawson does not. (2) Both $3(\alpha)$,11-dihydroxy-12-ketocholanic acids epimeric at C-11 yield $3(\alpha)$,11(α)-dihydroxycholanic acid among the products of Wolff-Kishner reduction, whereas this substance is not formed in appreciable amounts when the Marker and Lawson acid is subjected to the same treatment. (3) Since the properties of the two $3(\alpha)$,11-dihydroxy-12-ketocholanic acids are consistent with the configurations assigned, *i.e.* the 11(β)-hydroxy derivative is resistant to acetylation despite the activating influence of the α-keto group, whereas the 11(α)-hydroxy acid is readily acetylated, it can be safely assumed that no unexpected rearrangement has occurred in their preparation. On the other hand, the Marker and Lawson acid is prepared by vigorous alkaline hydrolysis of the 11-bromo-12-keto acid, under which conditions the maximum opportunity is offered for re-

TABLE I

Acetylated Esters of $3(\alpha)$,11-Dihydroxy-12-ketocholanates and of the Marker and Lawson Acid

Compound	M.p.	$[\alpha]_D$
	°C.	*degrees*
Methyl $3(\alpha)$,11(α)-diacetoxy-12-ketocholanate*...................................	153–154	+38
Methyl $3(\alpha)$,11(β)-diacetoxy-12-ketocholanate†....................................	109–110	+124
Methyl $3(\alpha)$,12(β)-diacetoxy-11-ketocholanate‡.................................	69	+54.5

* Data of Gallagher and Hollander (6).
† Data of Long and Gallagher (5).
‡ Data of Wintersteiner, Moore, and Reinhardt (7).

arrangement of the ketol structure. (4) Both 11-bromo-12-keto acids are converted to the Marker and Lawson acid by heating with strong alkali. Since these two bromo keto acids yield different products when hydrolyzed by alkali at room temperature, it is most probable that a rearrangement has occurred with the Marker and Lawson acid.

Since the ketone group must be either at C-11 or C-12 in Ring C, the rearrangement product is almost certainly 11-keto-3,12-dihydroxycholanic acid. This conclusion is supported by the results obtained when the Marker and Lawson acid is heated with hydrazine and sodium ethylate at 200°. The principal products of the reaction are a mixture of 3,11,12-trihydroxycholanic acids together with smaller amounts of lithocholic and lithocholenic acids. It would thus appear that both Longwell and Wintersteiner (2) and Marker, Shabica, Jones, Crooks, and Wittbecker

(3) were partly correct concerning the substances isolated from the Wolff-Kishner reduction.

The work reported in this paper was undertaken before the structure of the Marker and Lawson acid was understood, and with the object of preparing a 3,11-dihydroxycholanic acid. Although this object was not realized, it was possible to characterize certain of the products obtained when the Marker and Lawson acid was heated with sodium ethylate at 200° in the presence of excess hydrazine. Wintersteiner, Moore, and Reinhardt (7) simultaneously attacked the same problem and the progress of the investigations in the two laboratories has been accompanied by a continuous exchange of results. It is a pleasure to acknowledge the friendly cooperation of the Squibb investigators.

EXPERIMENTAL[1]

3(α),12(β)-Dihydroxy-11-ketocholanic Acid from Methyl 3(α)-Acetoxy-11-(β)-bromo-12-ketocholanate—3.00 gm. of methyl 3(α)-acetoxy-11(β)-bromo-12-ketocholanate were refluxed for 20 minutes with 25 ml. of 20 per cent potassium hydroxide in methanol. The solution was diluted with water and poured slowly into cold dilute sulfuric acid. The precipitate was washed thoroughly with water, dried, and recrystallized from 95 per cent ethanol. A total of 1.67 gm. melting at 200° was obtained in three crops for a yield of 72 per cent of theory. Upon admixture with a specimen of "3(α),11-dihydroxy-12-ketocholanic acid" (m.p. 206°), kindly given to us by Dr. O. Wintersteiner, the melting point was 201–205°. The acid was converted to the methyl ester with diazomethane. After recrystallization from methanol the methyl ester melted sharply at 155–156° and gave no depression with an authentic sample.

3(α),12(β)-Dihydroxy-11-ketocholanic Acid from Methyl 3(α)-Acetoxy-11(α)-bromo-12-ketocholanate—1.79 gm. of methyl 3(α)-acetoxy-11(α)-bromo-12-ketocholanate (m.p. 157–161°) were dissolved in 55 ml. of 20 per cent KOH in 90 per cent ethanol and heated under a reflux for 1 hour. The product was isolated as in the previous experiment. 837 mg. melting at 199–201° were obtained in the first two crops or a yield of 64 per cent of theory. The product gave no depression with an authentic sample. One recrystallization from ethyl acetate raised the melting point to 201–204°.

3(α),12(β)-Dihydroxy-11-ketocholanic acid was also prepared by vigorous alkaline hydrolysis of 3(α),11(β)-dihydroxy-12-ketocholanic acid.

Wolff-Kishner Reduction of 3(α),12(β)-Dihydroxy-11-ketocholanic Acid—

A mixture of 6.0 gm. of 3(α),12(β)-dihydroxy-11-ketocholanic acid and 6.0 ml. of 100 per cent hydrazine hydrate was heated overnight at 100° in an open glass bomb tube, protected by a $CaCl_2$ tube. A solution of sodium ethylate containing 4 gm. of sodium in 55 ml. of absolute ethanol was added, and the tube sealed and heated at 200° for 10½ hours. The contents were rinsed into an excess of hydrochloric acid and the acids extracted with ether. The crude acids were esterified with diazomethane and the oily esters were dried in a vacuum oven at 50° for 14 hours. The esters were chromatographed over 100 gm. of aluminum oxide and separated into four fractions by successive elution with ether (Fraction 1), ether-ethyl acetate, 1:1 (Fraction 2), ethyl acetate (Fraction 3), and with methanol (Fraction 4).

Methyl 3(α)-Hydroxy-Δ^{11}-cholenate (?)—Fraction 1 contained 1.44 gm. of solids which crystallized readily from 90–100° petroleum ether and melted at 94–97°. The product gave no depression when mixed with an authentic specimen of methyl 3(α)-hydroxy-Δ^{11}-cholenate. The weight of crystalline material obtained from this crop was 925 mg. or 17.2 per cent of theory. For further characterization, the compound was acetylated in pyridine with acetic anhydride. The acetylated methyl ester melted at 121–124° and gave no depression when mixed with an authentic specimen of methyl 3(α)-acetoxy-Δ^{11}-cholenate. When mixed with methyl 3(α)-acetoxycholanate (m.p. 135.5–137°), the melting point was 124–128°.

Fraction 2, eluted from the column with ether-ethyl acetate, 1:1, contained 1.40 gm. of solids and failed to crystallize. It was therefore dissolved in ether and rechromatographed over 20 gm. of aluminum oxide. The column was eluted with ether (500 ml.), with ether-ethyl acetate, 1:1 (600 ml.), and with ethyl acetate (500 ml.). From the ether eluate, 580 mg. of semicrystalline solids were obtained. These were combined with the non-crystalline residues from Fraction 1 (450 mg.) and acetylated by heating with pyridine and acetic anhydride. 634 mg. (10 per cent of theory). of crystals melting at 117–120° were obtained, which showed no depression when mixed with methyl 3(α)-acetoxy-Δ^{11}-cholenate. The total yield was 27 per cent of theory. In all probability, this product is a mixture of Δ^{11}-lithocholenic acid and lithocholic acid, since the melting point of the acetylated methyl ester is slightly higher than that of the purest sample of authentic methyl 3(α)-acetoxy-Δ^{11}-cholenate (118–120°). The reduction of 3(α),11(β)-dihydroxy-12-ketocholanic acid (8) and 3(α),11(α)-dihydroxy-12-ketocholanic acid (6) yielded these same two substances which form mixed crystals (9).

The ether-ethyl acetate eluates and the ethyl acetate eluates proved impossible to crystallize and were not further investigated.

3(α),11(α),12(α)-Trihydroxycholanic Acid—Fraction 3 contained 858 mg. and failed to crystallize readily. It was saponified with N NaOH

at room temperature and the acids isolated by acidification and thorough washing with water. The crude acids were crystallized from ethyl acetate and a product which melted at 131° was obtained. After two recrystallizations from the same solvent, it melted at 173–175° with preliminary softening at 145–165°; $[\alpha]_D^{31} = +31°$ (absolute ethanol).

$C_{24}H_{40}O_5$. Calculated, C 70.55, H 9.87; found (J. A.), C 70.28, H 9.84

Fraction 4, obtained from the column with methanol, weighed 2.14 gm. and did not crystallize readily. It was saponified with N NaOH at room temperature and the acids isolated by precipitation with acid. These were washed, dried, and recrystallized from ethyl acetate. The product from the first crystallization weighed 524 mg. and melted at 133° with bubbling. After four recrystallizations from ethyl acetate, the air-dried product melted at 135–140° with bubbles. After 6 hours drying at 100° in a good vacuum the compound melted at 170–172° with preliminary sintering at 140°; $[\alpha]_D^{31} = +27°$ (absolute ethanol). The compound was either a hydrate of $3(\alpha),11(\alpha),12(\alpha)$-trihydroxycholanic acid or was slightly impure, because when mixed with the acid obtained from Fraction 3 there was a slight depression of the melting point to 168–172°.

Methyl $3(\alpha),11(\alpha),12(\alpha)$-Triacetoxycholanate—116 mg. of $3(\alpha),11(\alpha),$-$12(\alpha)$-trihydroxycholanic acid were esterified with diazomethane and the product acetylated by heating for 2 hours with acetic anhydride and pyridine. The ester was isolated in the usual manner, freed from traces of colored impurity by passage over aluminum oxide, and crystallized from dilute methanol. After one recrystallization from this solvent and one recrystallization from 90–100° petroleum ether the compound melted at 163.5–165°; $[\alpha]_D^{31} = +33°$ (chloroform).

$C_{31}H_{48}O_8$. Calculated, C 67.85, H 8.82; found (J. A.), C 67.81, H 8.72

Methyl $3(\alpha),11(\alpha),12(\beta)$-Triacetoxycholanate—The non-crystalline residues from Fractions 3 and 4 were combined and esterified with diazomethane. The esters were acetylated with acetic anhydride and pyridine by heating under a reflux for 14 hours, and the reaction product diluted with water and extracted with ether. After removal of the ether, the residue crystallized from ethyl acetate. 200 mg. of a product melting at 181–185° were obtained in the first crop. After two recrystallizations from 90–100° petroleum ether it formed long needles melting at 188–189.5°; $[\alpha]_D^{31} = +54°$ (chloroform).

$C_{31}H_{48}O_8$. Calculated, C 67.86, H 8.82; found (J. A.), C 67.68, H 8.80

The mother liquor from the first crop yielded 745 mg. of crystals from 0–100° petroleum ether. These softened from 119–149° and gave a clear melt at 161°. Fractional crystallization from petroleum ether yielded

125 mg. of triacetate methyl ester melting at 188–189.5° and 389 mg. of a compound melting at 150–153°. Two recrystallizations of the latter from dilute methanol gave a product which melted sharply at 163.5–165°, $[\alpha]_D^{31} = +33°$ (chloroform), and showed no depression when mixed with methyl $3(\alpha), 11(\alpha), 12(\alpha)$-triacetoxycholanate.

$3(\alpha), 11(\alpha), 12(\beta)$-*Trihydroxycholanic Acid*—226 mg. of methyl $3(\alpha),$-$11(\alpha), 12(\beta)$-triacetoxycholanate were dissolved in 2.5 ml. of methanol, 2.5 ml. of 2.93 N KOH added, and the mixture heated under a reflux for 3 hours. 1.727 milliequivalents of alkali were consumed; calculated for $C_{31}H_{48}O_8$ (4 equivalents), 1.648 ml. The acid, isolated by acidification and extraction with ether, crystallized from ethyl acetate in needles which after two recrystallizations from the same solvent melted at 164–166°; $[\alpha]_D^{26} = +45°$ (absolute ethanol).

$C_{24}H_{40}O_5$. Calculated, C 70.55, H 9.87; found (J. De L.), C 70.47, H 9.76

$3(\alpha), 11(\beta), 12(\alpha)$-*Trihydroxycholanic Acid*—In a second experiment in which 1.55 gm. of $3(\alpha), 12(\alpha)$-dihydroxy-11-ketocholanic acid were reduced with hydrazine and sodium ethylate, the three acids already described were obtained in similar yields by essentially the same procedures. In addition, however, Fractions 2 and 3 eluted respectively from aluminum oxide with ethyl acetate-ether, 1:1, and ethyl acetate upon saponification yielded an acid which crystallized in prisms from ethyl acetate. The product melted at 140° and foamed at 165–170°. After three recrystallizations from ethyl acetate the air-dried product melted at 138° and formed bubbles at 143–145°. After 6 hours drying at 90° in a high vacuum, it sintered weakly at 139–140° and melted at 147–149° with some indication of bubbling; $[\alpha]_D^{26} = +54°$ (absolute ethanol).

$C_{24}H_{40}O_5$. Calculated, C 69.94, H 9.90; found (J. A.), C 69.85, H 9.63

The amount of material obtained was too small for further characterization.

Methyl $3(\alpha), 11(\beta), 12(\alpha)$-Triacetoxycholanate[2]—1.675 gm. of $3(\alpha), 12(\alpha)$-dihydroxy-11(β)-acetoxycholanic acid, prepared by the method of Long and Gallagher (5), were esterified with diazomethane and heated under a reflux for 2 hours with 3.5 ml. of acetic anhydride in 20 ml. of pyridine. The ester was isolated in the usual manner and crystallized from petroleum ether. 1.62 gm. were obtained, melting at 123–124°. The product was freed from a small amount of colored material by passage through a short column of aluminum oxide and after three recrystallizations large prisms which melted at 129–130° were obtained; $[\alpha]_D^{25} = +51°$ (chloroform).

$C_{31}H_{48}O_8$. Calculated, C 67.86, H 8.82; found (J. De L.), C 67.81, H 9.03

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[2] Prepared by Miss Evelyn Borgstrom.

3(α),11(β),12(α)-Trihydroxycholanic Acid[2]—1.57 gm. of methyl 3(α),-11(β),12(α)-triacetoxycholanate were hydrolyzed by boiling for 4 hours under a reflux with 30 ml. of methanol and 75 ml. of 2 N NaOH. The solution was acidified and extracted with ether. The acid crystallized from ethyl acetate as prisms which softened at 121° and melted with bubbles at 122–125°. Four recrystallizations from ethyl acetate yielded clusters of needles which on standing changed to prisms. These sintered slightly at 129–130° and melted at 145–146° after thorough drying at 100° in a high vacuum; $[α]_D^{26} = +54°$ (ethanol).

DISCUSSION

It is clear from the experimental results that the principal products obtained when the Marker and Lawson acid, $3(α),12(β)$-dihydroxy-11-ketocholanic acid, is heated with hydrazine and sodium ethylate are $3(α),-11,12$-trihydroxycholanic acids and smaller amounts of substances in which both oxygen atoms in Ring C have been eliminated. It is doubtful whether the reduction can be considered a Wolff-Kishner reaction of the abnormal type described by Dutcher and Wintersteiner (4), since the initial formation of a hydrazone has not been demonstrated. It is far more likely that the trihydroxy acids are formed by the reducing action of hydrazine and its decomposition products in the alkaline solution. In order to account for the products in which oxygen is eliminated from Ring C it must be assumed that the 11-keto-12-hydroxy acid is in equilibrium with an 11-hydroxy-12-keto acid which can form a hydrazone; subsequent reduction of this group results in a product unsaturated in Ring C. This explanation would assume that a reaction analogous to that postulated by Gallagher and Long (8) for the reduction of $3(α),11(β)$-dihydroxy-12-ketocholanic acid had taken place; *i.e.*, that $3(α),11$-dihydroxycholanic acid is formed as an intermediate and that elimination of the 11-hydroxyl group occurs as in the accompanying partial formulations. In this work the monohydroxy acids were not separated from each other although they were undoubtedly a mixture, as noted in the experimental section. Gallagher and Long (8) have shown that the products obtained from the Wolff-Kishner reduction of $3(α),-11(β)$-dihydroxy-12-ketocholanic acid were a mixture of $Δ^{11}$-lithocholenic acid and lithocholic acid in which $Δ^{9,11}$-lithocholenic acid was either absent or present only in traces. Similar results were obtained by Gallagher and Hollander (6) in the reduction of $3(α),11(α)$-dihydroxy-12-ketocholanic acid. If the explanation of these authors is correct, a similar mixture, but in a smaller yield, is formed in this reaction. Likewise, some $3(α),11(α)$-dihydroxycholanic acid should have been formed. Although it was not isolated from these experiments nor was it observed by Wintersteiner, Moore, and Reinhardt (7), it may have been present in small amounts and

escaped isolation. It is certain, however, that it does not constitute any considerable portion of the reaction product.

The four possible 3,11,12-trihydroxycholanic acids stereoisomeric at C-11 and C-12 have been isolated as a result of this investigation and that of Wintersteiner, Moore, and Reinhardt (7). They are described in Table II. The·products which correspond most closely with the compound described by Marker, Shabica, Jones, Crooks, and Wittbecker (3) are either $3(\alpha),11(\beta),12(\alpha)$-trihydroxycholanic acid (m.p. 145–147°; $[\alpha]_D = +54°$) or the hydrate of $3(\alpha),11(\alpha),12(\alpha)$-trihydroxycholanic acid (m.p., air-dried product, 135–140° with bubbles; $[\alpha]_D = +27°$). Unfortunately the rotation was not recorded by these investigators.

The configuration of the four trihydroxycholanic acids can be ascertained only by consideration of both the results of this investigation and that of Wintersteiner, Moore, and Reinhardt (7). The latter authors have provided evidence for the configuration of $3(\alpha),11(\alpha),12(\alpha)$-trihydroxycholanic acid by the preparation of this compound from Δ^{11}-lithocholenic acid with osmium tetroxide. This result establishes the configuration of the two hydroxyl groups in Ring C as either $11(\beta),12(\beta)$ or $11(\alpha),(12(\alpha)$. Since these authors have shown that the compound is completely acetylated and saponified quite readily, there can be little doubt that the C-11 hydroxyl group is in the α configuration, since an $11(\beta)$-hydroxyl is not easily acetylated and has a very marked tendency to be eliminated from the molecule.

The configuration of $3(\alpha),11(\beta),12(\alpha)$-trihydroxycholanic acid has been established through its preparation by acetolysis of the oxide of Δ^{11}-lithocholenic acid. The chemical behavior of the product and its derivatives described in Paper II (10) are in agreement with the configuration which has been assigned.

The two possible structures for the remaining two 3,11,12-trihydroxycholanic acids are $3(\alpha),11(\alpha),12(\beta)$ and $3(\alpha),11(\beta),12(\beta)$. A decision on the configuration of these two isomers can be made, since one of them forms a methyl ester triacetate melting at 188–189.5°. The ready introduction of an acetoxy group at C-11 is possible only if the hydroxyl group is in the α or unhindered configuration; i.e., *trans* to the angle methyl group at C-10. Moreover, Wintersteiner, Moore, and Reinhardt (7) have shown that methyl $3(\alpha),11(\alpha),12(\beta)$-trihydroxycholanate can be converted to a diacetoxy monohydroxy ester by acetylation with acetic anhydride and pyridine at room temperature. Oxidation of this deriva-

TABLE II

Isomeric $3(\alpha),11,12$-Trihydroxycholanic Acids

Compound	M.p.	$[\alpha]_D$
	°C.	degrees
$3(\alpha),11(\alpha),12(\alpha)$-Trihydroxycholanic acid...	173–175	+31
$3(\alpha),11(\alpha),12(\beta)$-Trihydroxycholanic " ...	164–166	+45
$3(\alpha),11(\beta),12(\beta)$-Trihydroxycholanic " *..	177	+43
$3(\alpha),11(\beta),12(\alpha)$-Trihydroxycholanic " ...	145–147	+54

* Data of Wintersteiner, Moore, and Reinhardt (7).

tive yielded methyl $3(\alpha),11(\alpha)$-diacetoxy-12-ketocholanate identical with the product obtained by Gallagher and Hollander (6), in Paper V of this series. There can therefore be little doubt that this compound has the α configuration at C-11, and since the possibility for an α configuration of the C-12 hydroxyl has already been excluded, it follows that the structure is $3(\alpha),11(\alpha),12(\beta)$-trihydroxycholanic acid.

The configuration of the remaining product which was not isolated in these experiments can be assigned by exclusion as $3(\alpha),11(\beta),12(\beta)$-trihydroxycholanic acid. The results of Wintersteiner, Moore, and Reinhardt (7) completely confirm this conclusion, since the product yielded only a diacetate methyl ester upon mild acetylation whereas it was destroyed by vigorous treatment with acetic anhydride in pyridine. This is consistent with the labile character of the $11(\beta)$-hydroxyl group. The diacetoxy monohydroxy methyl ester, however, upon oxidation with CrO_3 was converted to the methyl ester diacetate of the Marker and Lawson acid.

This important result furnishes additional proof for the 11-keto structure assigned on the basis of our experiments and furthermore establishes the configuration of the C-12 hydroxyl group in the Marker and Lawson acid. This finding likewise confirms the assumption that the molecule has undergone no extensive rearrangement.   It can therefore be concluded that this fourth isomer has the structure $3(\alpha),11(\beta),12(\beta)$-trihydroxycholanic acid and that the configuration of the Marker and Lawson acid is $3(\alpha),12(\beta)$-dihydroxy-11-ketocholanic acid.

I am indebted to Miss Joanna Xenos for technical assistance and to Miss Evelyn Borgstrom for the preparation of two compounds.

SUMMARY

1. The "3,11-dihydroxy-12-ketocholanic acid" of Marker and Lawson is actually $3(\alpha),12(\beta)$-dihydroxy-11-ketocholanic acid.

2. When heated with hydrazine and sodium ethylate at 200° this compound is converted in part to lithocholic and lithocholenic acids and in part to four diastereoisomeric 3,11,12-trihydroxycholanic acids.   Three of these acids were isolated in this investigation.

3. Configurations have been assigned to the four 3,11,12-trihydroxycholanic acids upon the basis of their reactions and their preparation by alternate methods.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

VII. DEGRADATION OF THE SIDE CHAIN OF CHOLANIC ACID*

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The stepwise degradation of the bile acid side chain by the Barbier-Wieland procedure is cumbersome, especially when large amounts of material must be manipulated, and in the majority of instances the yields are far from satisfactory (1–4). Removal of the side chain by oxidation in one step with CrO_3, as in the familiar degradation of the cholesterol side chain (5), when applied to the bile acids yields very small amounts of etio acid. The neutral products of oxidation are difficult to separate and are likewise obtained in small yield (6).[1] The work recorded here was begun as a model experiment in the hope that a procedure could be devised in which the side chain of the bile acids could be degraded by 2 carbon atoms without the necessity of using the Grignard reaction. Although this end was attained, the yield encountered in the final stages was unexpectedly poor and at the present time it would appear that the method is unsatisfactory for the removal of the side chain of the bile acids. At the completion of this work a preliminary report of the experiments of Jacobsen (7) appeared. The procedure of these authors, using the phenyl ketone instead of the methyl ketone, seems to offer certain advantages which were not achieved in the present work.[2]

The procedure we have investigated is formulated in the accompanying diagram. Several complications were encountered. The bromo ketone (VI) proved to be difficult to purify. Whether this is caused by traces of 25-bromo ketone or by the presence of the two possible epimeric 23-bromo ketones has not been decided. The dehydrobromination of VI was possible

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[1] Personal communications from other members of the group and unpublished experiments from this laboratory.

[2] The promising procedures of Meystre, Frey, Wettstein, and Miescher (8) and of Meystre, Ehmann, Neher, and Miescher (9) were received while this manuscript was in press.

with boiling collidine but the α,β-unsaturated ketone was obtained only as an oil. The oxidation of VII by CrO_3 gave poor yields of VIII. The characterization of the final product was difficult because of the several isomeric bisnorcholanic acids described in the literature (1).

<div align="center">EXPERIMENTAL[3]</div>

25-Chloromethyl Norcholanyl Ketone (IV)—9.0 gm. of cholanic acid (I) were dissolved in 25 ml. of thionyl chloride purified by the procedure of Glattfeld and Kribben (10) and the solution allowed to stand at room temperature for 4 hours. The excess thionyl chloride was distilled under diminished pressure on the steam bath and the removal completed by the distillation of fresh portions of benzene from the crystalline mass. The

acid chloride was dissolved in 100 ml. of benzene and with continuous stirring and cooling to $-10°$ added dropwise to a large excess of ethereal diazomethane, previously dried with sodium wire. The reaction mixture stood overnight, during which time the temperature was permitted to rise to that of the room. The diazo ketone failed to crystallize readily and even after chromatographing 1.08 gm. on Al_2O_3 no crystalline product was obtained. The remainder of the diazo ketone was therefore treated with 300 ml. of a 3.5 N solution of hydrogen chloride in anhydrous ether and the reaction mixture allowed to stand for 10 minutes. The ether was washed

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with dilute Na_2CO_3 solution and with water, dried over Na_2SO_4, and the ether removed. 9.0 gm. of crude crystalline product were obtained. This was purified by chromatographing on 60 gm. of Al_2O_3 and 6.1 gm. of product obtained which after two recrystallizations from methanol and from acetone gave needles with a melting point of 109–110°; $[\alpha]_D^{25} = +21.7°$ (CHCl₃).

$C_{25}H_{41}OCl$.   Calculated.   C 76.40, H 10.51, Cl 9.02
        Found (J. A.).   " 76.29, " 10.51, " 9.41

Norcholanyl Methyl Ketone—4.1 gm. of slightly impure 25-chloromethyl norcholanyl ketone were dissolved in 20 ml. of glacial acetic acid, 10 gm. of granulated zinc added, and the mixture heated under a reflux for 2 hours. The solution was cooled, ether added, and the zinc removed by decantation. The ether solution was extracted with $NaHCO_3$ solution and with water and the ether removed. 3.6 gm. of crude product were obtained, which was purified by chromatographic adsorption on Al_2O_3. 1.98 gm. of analytically pure material were obtained together with smaller quantities of colored crystalline product which were discarded. Recrystallized from acetone and from methanol the compound formed long thin needles, m.p. 114–115°; $[\alpha]_D^{27} = +22.5°$ (CHCl₃).

$C_{25}H_{42}O$.   Calculated, C 83.73, H 11.78; found (J. A.), C 83.30, H 11.64

23-Bromonorcholanyl Methyl Ketone—1.65 gm. of norcholanyl methyl ketone were dissolved in 50 ml. of glacial acetic acid, and 15.2 ml. of 0.130 N Br_2 in glacial acetic acid and 2 drops of 48 per cent aqueous hydrobromic acid added. After 1 hour the reaction mixture was diluted with water and extracted with ether. The ether solution was washed with $NaHCO_3$ solution and with water and the ether removed. The residue was purified by chromatographic adsorption on alumina.[4] 1.97 gm. of a light oil were

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[4] In earlier experiments with this substance and 25-bromomethyl norcholanyl ketone chromatographic adsorption had been used in purification. Considerable difficulty was experienced in obtaining satisfactorily pure preparations and the halogen values were invariably low. Mr. W. Saschek, Department of Biochemistry, Columbia University, called our attention to his observation that the silver halide precipitate obtained in his microanalysis appeared to be silver chloride rather than silver bromide; analysis of the silver salt confirmed this observation. We were at that time using an alumina which had been extracted with 10 per cent hydrochloric acid and subsequently washed twenty times with distilled water. Although the final seven washings were neutral, the alumina still contained considerable chloride ion. An exchange reaction apparently occurred on the column. This possibility is of importance when sensitive substances are chromatographed. We have since substituted acetic acid in the extraction of base from commercial alumina and have not experienced any similar difficulty. We should like to express our appreciation for the assistance of Mr. Saschek.

eluted with 60–70° petroleum ether and crystallized from methanol. It proved difficult to obtain a product with a sharp melting point and seven crystallizations were required before long needles of constant melting point, 94.5–95.5°, were obtained; $[\alpha]_D^{28} = +86.8°$ (CHCl₃).

$C_{25}H_{41}OBr$. Calculated.   C 68.63, H 9.45, Br 18.27
Found (T. S. M.). " 69.09, " 9.05, " 18.37

Methyl Bisnorcholanate and Bisnorcholanic Acid—2.38 gm. of 23-bromonorcholanyl methyl ketone were heated under a reflux for 4 hours with 5.0 ml. of redistilled collidine. After cooling, ether was added and the solution washed with dilute HCl and with water, and the ether removed. The dark gummy residue was dissolved in petroleum ether and chromatographed over 30 gm. of alumina. The eluate from 500 ml. of petroleum ether consisted of 1.20 gm. of a light yellow oil. Attempts to crystallize this product were unavailing. It was dissolved in 45 ml. of glacial acetic acid, 50 ml. of 1.36 N CrO₃ were added, and the mixture was allowed to stand for 18 hours at room temperature. The excess CrO₃ was destroyed by the addition of methanol and the product separated into acid and neutral fractions. The neutral fraction weighed 517 mg. The acid fraction (174 mg.) was esterified with diazomethane and purified by chromatographic separation on alumina. 29 mg. of feathery crystals were obtained which upon recrystallization from ethanol melted at 115–117°; $[\alpha]_D^{26} = +22.6°$ (CHCl₃); $[\alpha]_D^{28} = +21.4°$ (95 per cent ethanol).

$C_{23}H_{38}O_2$. Calculated, C 79.83, H 11.07; found (J. De L.), C 79.32, H 11.15

The methyl ester was saponified with 0.05 N alcoholic NaOH under a reflux and the acid obtained crystallized from petroleum ether as small prisms, m.p. 171–175°; $[\alpha]_D = +10°$ (95 per cent ethanol). For the ε isomer of bisnordesoxycholic acid Wieland, Schlichting, and Jacobi (1) report the melting point of the free acid as 181°, $[\alpha]_D^{27} = +14°$, and the methyl ester melting point 117°.

In a subsequent experiment isolation of the bisnorcholanic acid was achieved by preliminary saponification of the methyl esters of the acidic fraction with alcoholic base at room temperature, since methyl bisnorcholanate is saponified with difficulty under these conditions. 674 mg. of acids were esterified with diazomethane. The product was dissolved in 15 ml. of 95 per cent ethanol; 1.0 ml. of 3.2 N NaOH was added and the solution allowed to stand at room temperature for 2 hours. 341 mg. of unhydrolyzed esters were obtained. These were completely hydrolyzed by heating under a reflux with alcoholic base and the acids isolated. 161 mg. were obtained which crystallized poorly from petroleum ether. The product was esterified with diazomethane and the ester recrystallized from

ethanol as needles, m.p. 65–66°; $[\alpha]_D^{25} = +9.1°$ (chloroform); $+30.3°$ (ethanol).

$C_{21}H_{18}O_2$. Calculated, C 79.83, H 11.07; found (A. J. H.-S.), C 79.97, H 11.04

The methyl ester was saponified with 0.05 N NaOH under a reflux and the acid obtained melted at 196–201°; $[\alpha]_D^{25} = +19.5°$ (ethanol). Wieland, Schlichting, and Jacobi (1) report the melting point of the β isomer of bisnorcholanic acid as 242°, $[\alpha]_D^{25} = +23°$, and the methyl ester of this isomer as an oil.

SUMMARY

A procedure for the degradation of the bile acid side chain by the elimination of 2 carbon atoms has been described. Cholanic acid was converted to norcholanyl methyl ketone, brominated at C-23, and the elements of HBr removed with collidine. Oxidation by CrO_3 yielded two isomers of bisnorcholanic acid.

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STEROIDS DERIVED FROM BILE ACIDS

I. THE PREPARATION OF 3(α)-HYDROXY-Δ11-CHOLENIC ACID FROM DESOXYCHOLIC ACID

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Reichstein and his associates (1) have recently reported the partial synthesis of dehydrocorticosterone. This achievement not only confirms the structure assigned to this hormone of the adrenal cortex but also provides a method for the partial synthesis of that group of hormones which have an atom of oxygen at C_{11}. However, the large number of steps required and the limited yields of certain intermediate compounds have restricted the commercial preparation of the hormone to such a degree that it still is not available in sufficient quantities for use on experimental animals and in clinical medicine. It is highly desirable that dehydrocorticosterone, corticosterone, and the two related derivatives with a hydroxyl group at C_{17} be made available. To this end an investigation was undertaken in the biochemical laboratory of the Mayo Foundation in 1940.[1]

The preparation of dehydrocorticosterone from desoxycholic acid involves four principal problems: removal of the oxygen at C_{12} and attachment of oxygen at C_{11}, degradation of the side chain, introduction of the ketol group on C_{20}, C_{21}, and conversion of the hydroxyl group at C_3 to an unsaturated ketone $C_3\Delta^4$. The work will be presented in the order given.

Although the investigation was quite independent of that of the Swiss workers, the original plan for introduction of the oxygen at C_{11} was the same, namely through an intermediate compound with a double bond Δ^{11} in Ring C of the steroid nucleus. Therefore it became necessary to devise a method for the preparation of compounds with this structure from some abundant source of starting material. Desoxycholic acid appeared to be the best starting material and for purposes of study $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid was chosen as the intermediate compound. A method for the preparation of this unsaturated acid from desoxycholic acid is given in this paper. Through a study of derivatives of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid a highly satisfactory method for introduction of oxygen at C_{11} was found. The essential intermediate compounds, which include some hitherto

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[1] A manuscript which described the preparation of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid was deposited with the Chairman of the Division of Chemistry and Chemical Technology, the National Research Council, Washington, D. C., in October, 1941.

unknown derivatives of cholanic acid, will be described in subsequent papers.

· In the preparation of 3(α)-hydroxy-Δ^{11}-cholenic acid, the double bond in Ring C was introduced by pyrolysis of methyl 12-benzoyldesoxycholate. The average yield from a number of preparations of the lithocholenic acid was 21 per cent of the starting material and was 45 per cent of the weight of the starting material not recovered.

The steroid nucleus was shown to be intact by reduction of the double bond with formation of lithocholic acid. The position of the double bond was shown by formation of an oxide with perbenzoic acid and reduction of the oxide to a hydroxyl group. Hydrogen and Adams' platinum oxide catalyst in acetic acid and a small amount of hydrochloric acid yielded desoxycholic acid. The oxide therefore was at C_{11}, C_{12} and in the presence of mineral acid rupture of the oxide resulted in formation of the 12-hydroxyl group.

DISCUSSION

Wieland and his associates (2–5) have prepared cholenic, choladienic, and cholatrienic acids by pyrolysis of lithocholic, desoxycholic, and cholic acids respectively. $3(\alpha)$-Hydroxy-Δ^{11}-cholenic acid was probably first obtained by Longwell and Wintersteiner (6) by the Wolff-Kishner method of reduction of what they considered to be 3,11-dihydroxy-12-ketocholanic acid. It was isolated in small yield as a by-product and was not identified. The melting point, 163°, however, indicates that it was probably the Δ^{11}-lithocholenic acid.

After the work described in this paper had been completed, Reichstein and his associates (7) reported the preparation of the desired acid through pyrolysis of methyl 3-keto-12-benzoxycholanate, followed by reduction of the ketone group to give a mixture of $3(\alpha)$- and (β)-hydroxy-Δ^{11}-cholenic acids. Pyrolysis of methyl 3-acetoxy-12-benzoxycholanate also gave some of the cholenic acid but in less amount. More recently Lardon and Reichstein (8) have obtained the 3-ketobisnorcholenic acid by pyrolysis of the anthraquinone-β-carboxylic ester of methyl 12-hydroxy-3-ketobisnorcholanate.

EXPERIMENTAL

Methyl Desoxycholate (II)—1 kilo of a commercial preparation of desoxycholic acid (I) was suspended in 4.5 liters of methanol and methanolic hydrogen chloride was added to make the solution 0.1 N. At room temperature the acid dissolved within 1 hour and esterification was complete within 2 hours, but the solution was usually allowed to stand overnight. Crystals of the ester which separated were dissolved by warming and the solution was treated with activated carbon and filtered through infusorial earth. Concentration of the methanol gave three successive crops of ester, 674 gm., m.p.[2] 82–88°, 231 gm., m.p. 72–78°, and 108 gm. of low melting point. The crystals were washed with cold methanol and dried at room temperature. The first two crops were used for subsequent treatment; the third crop and mother liquor were converted into desoxycholic acid

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[2] All melting points were taken on the Fisher-Johns apparatus.

and purified.   Dane and Brady (9) have shown that methyl desoxycholate crystallizes from methanol with 1 mole of methanol for each 2 moles of ester.

Methyl 3-Benzoyldesoxycholate (III)—406 gm. of methyl desoxycholate (II) were dissolved in 1500 cc. of dry benzene and 300 to 400 cc. of the benzene were boiled off at atmospheric pressure to remove methanol. 400 cc. of dry benzene, 100 cc. of pyridine, and 125 cc. of benzoyl chloride were added and the flask was allowed to stand at room temperature overnight.   The mixture was washed with dilute hydrochloric acid, with a solution of sodium bicarbonate, and with water and was concentrated under reduced pressure to a thick syrup to which 800 cc. of ethyl ether were added.   Crystals of methyl 3-benzoyldesoxycholate separated, combined with ether in the proportion of 2 moles of ester to 1 mole of ether.   The yield was 472 gm., m.p. 90–95° and $[\alpha]_D = +49° \pm 1°$ (1.6 per cent in chloroform); $[\alpha]_D = +61° \pm 1°$ (1.6 per cent in methanol).

Dried in a vacuum at 100° for 4 hours
$C_{32}H_{46}O_5$.   Calculated, C 75.25, H 9.08; found, C 75.11, H 9.26
Dried in air at room temperature
$C_{32}H_{46}O_5 \cdot \frac{1}{2}(C_2H_5)_2O$.   Calculated, C 74.75, H 9.40; found, C 74.35, H 9.40

Methyl 3,12-Dibenzoyldesoxycholate (IV)— The dibenzoyl derivative was prepared without separation of the methyl 3-benzoyldesoxycholate in crystalline form.   The residue, after removal of benzene in the previous preparation, was dissolved in 300 cc. of pyridine to which 150 cc. of benzoyl chloride were added.   The flask was heated at 100° for 1 hour, pyridine was removed under reduced pressure, and to the residue 1 liter of water and 1 liter of benzene were added.   The benzene solution was washed with hydrochloric acid, with a solution of sodium bicarbonate, and with water.

The benzene was removed under reduced pressure, 1 liter of ethanol was added, and the solution was concentrated under reduced pressure.   The mixture was allowed to crystallize overnight and the crystals were filtered, washed with cold ethanol, and dried at room temperature.   The weight of dry material was 491 gm. and the melting point was 143–143.5°. $[\alpha]_D = +97° \pm 1°$ (1.6 per cent in chloroform); $[\alpha]_D = +122° \pm 2°$ (0.8 per cent in methanol).

Analysis—$C_{39}H_{50}O_6$.   Calculated, C 76.18, H 8.20; found, C 76.15, H 8.00

12-Benzoyldesoxycholic Acid (V)—307 gm. (0.5 mole) of methyl 3,12-dibenzoyldesoxycholate and 220 cc. of 5 N aqueous sodium hydroxide were added to 1800 cc. of 95 per cent ethanol.   The mixture was boiled under a reflux for 30 minutes, cooled to room temperature, and filtered through

infusorial earth. To the clear filtrate 200 to 230 cc. of 5 N hydrochloric acid were added and ethanol was removed under reduced pressure until cloudiness appeared. Separation of crystalline material in a small sample was induced by the addition of a few drops of water. These seed crystals were returned to the flask and after a heavy crop of crystals had separated 1000 cc. of water were added gradually while the contents of the flask were stirred with a mechanical stirrer. The crystals were filtered after 20 minutes. If the solution was allowed to stand, benzoic acid contaminated the product. The precipitate was heated in 1 liter of water at 80° for 1 hour to remove benzoic acid. It was then filtered and air-dried. The weight was 243 gm. and the melting point 182.5–183°. $[\alpha]_D = +51° \pm 2°$ (1.6 per cent in chloroform); $[\alpha]_D = +65° \pm 2°$ (1.6 per cent in methanol).

Analysis—$C_{31}H_{44}O_5$. Calculated, C 74.94, H 8.93; found, C 74.22, H 8.92

Methyl 12-Benzoyldesoxycholate (VI)—209 gm. (0.41 mole) of 12-benzoyldesoxycholic acid and 37 cc. of concentrated sulfuric acid were added to 1900 cc. of methanol and the solution was refluxed overnight.[3] Under reduced pressure 1500 cc. of methanol were removed and to the residue 1500 cc. of benzene were added. The benzene solution was washed with water, with a solution of sodium bicarbonate, and with water. It was dried over Na_2SO_4 and concentrated under reduced pressure. The last traces of benzene were removed in the special distilling flask used in the pyrolysis. The yield of the methyl ester was assumed to be quantitative. Methyl 12-benzoyldesoxycholate has not been separated in crystalline form.

3(α)-Hydroxy-Δ¹¹-cholenic Acid (VII)—The methyl 12-benzoyldesoxycholate described in the preceding paragraph was placed in a special flask of 500 cc. capacity.[4] The flask was attached to a water suction pump and immersed in a steam bath; after about 2 hours only traces of benzene remained. A thermometer was placed in the extended neck and rested on the bottom of the flask. The flask attached to the water pump was then placed in an air bath which was previously heated to 300°. As soon as the temperature inside the flask reached 300°, benzoic acid began to distil. The time required for the temperature to reach 300° was less than 3 minutes

[3] It was later found that the acid was esterified in 4 parts of methanol with 0.2 N hydrochloric acid at room temperature in less than 2 hours.

[4] The neck of the flask was lengthened to 46 cm. from the bottom of the flask. The opening at the end of the neck was constricted to 15 mm. and was closed with a rubber stopper. The diameter of the neck was 25 mm. A side tube of the same diameter and 50 cm. long was attached to the neck 38 cm. above the bottom of the flask. The end of the side arm was constricted to 15 mm. and closed with a rubber stopper. Suction was applied to the flask through an opening 5 cm. from the end of the side arm. The side arm was maintained sufficiently cool to permit the benzoic acid to crystallize as it was liberated during the pyrolysis.

and to reach 315° less than 5 minutes.   25 minutes after the temperature reached 315° heating was stopped and the flask was cooled with a blast of air.   When the temperature was below 140° the vacuum was released and the viscous, almost colorless residue was dissolved in 2600 cc. of ethanol to which 260 cc. of 5 N aqueous sodium hydroxide were added.   The alkaline solution was boiled under a reflux for 2 hours, the ethanol was removed, and the aqueous alkaline solution was acidified with hydrochloric acid.   The precipitated acid was filtered and washed until free of chlorides.

The crude acid was dissolved in 2 liters of hot benzene, occluded water was separated, and, after cooling, 12-benzoyldesoxycholic acid which separated was removed by filtration.[5]

The separation of the lithocholenic acid from 12-benzoyldesoxycholic acid was based on the observation that when these acids were dissolved in benzene the lithocholenic acid formed an insoluble sodium salt with an aqueous solution of sodium bicarbonate but the 12-benzoyldesoxycholic acid remained in the benzene.

The benzene solution of the two acids was concentrated to a volume of 2 liters to which was added a solution of 130 gm. of sodium bicarbonate in 2 liters of water.   The mixture was refluxed gently for 3 hours and the benzene layer was decanted while hot.   The aqueous layer was washed with benzene.   A small amount of 12-benzoyldesoxycholic acid was recovered from the benzene.   The aqueous solution was cooled and the sodium salt was filtered off and washed with saturated, cold sodium bicarbonate solution.

The sodium salt of the lithocholenic acid was dissolved in 1200 cc. of boiling water and the free acid was precipitated with hydrochloric acid. The lithocholenic acid was filtered off, dissolved in benzene, and again treated with an aqueous solution of sodium bicarbonate.   The sparingly soluble sodium salt of the acid was heated at 100° overnight with 600 cc. of N sodium hydroxide and was filtered through a steam-jacketed Büchner funnel.   The sodium salt of the lithocholenic acid remained undissolved in the normal sodium hydroxide; the impurities passed into the filtrate (the last traces of 12-benzoyldesoxycholic acid were converted to sodium desoxycholate).

The sodium salt of the lithocholenic acid was dissolved in 1200 cc. of boiling water and the acid was precipitated with hydrochloric acid, filtered, dried, and crystallized from benzene.   The purified acid (10), m.p. 165–165.5°, weighed 32 gm. $[\alpha]_D = +43° \pm 2°$ (3 per cent in ethanol); $[\alpha]_D = +31° \pm 2°$ (1 per cent in chloroform).   The weight of 12-benzoyldesoxy-

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[5] The recovered crude 12-benzoyldesoxycholic acid was recrystallized from benzene and digested with hot water to change the crystal form.   The suspension was filtered, dried, and reesterified.

cholic acid recovered was 110 gm. The yield of lithocholenic acid based on the 12-benzoyldesoxycholic acid not recovered was 45 per cent.

Analysis—$C_{24}H_{38}O_3$. Calculated, C 76.94, H 10.23; found, C 76.77, H 10.30

Lithocholic Acid from 3(α)-Hydroxy-Δ^{11}-cholenic Acid—5 gm. (0.0134 mole) of 3(α)-hydroxy-Δ^{11}-cholenic acid were reduced in 50 cc. of glacial acetic acid in the presence of 50 mg. of platinum oxide. 301 cc. of hydrogen were absorbed in 56 minutes; theory for 1 mole of hydrogen, 300 cc. Crystals which had separated from the solution were dissolved by warming, the platinum was removed, and the acetic acid was concentrated under reduced pressure to about 10 cc. Crystallization from acetic acid yielded 3.54 gm. of material, which had a melting point of 179–182°. The melting point was raised to 184–185° by treatment with boiling benzene (50 cc. per gm.). 2 gm. of the acid were esterified with diazomethane. The ester was crystallized from acetic acid. The melting point was 126–127°. An authentic sample of methyl lithocholate prepared from 3(α)-hydroxy-12-ketocholanic acid by reduction with the Wolff-Kishner method melted at 128–128.5° (11); the melting point of the mixture was 126–128°. 1 gm. of ester was oxidized with 10 cc. of 1.98 N chromic acid in acetic acid for 1 hour at room temperature. The oxidized product was crystallized from acetic acid; it melted at 116–118°. An authentic sample of methyl dehydrolithocholate melted at 117–119° (12). The melting point of the mixture was 116–119°.

3(α)-Hydroxy-11,12-epoxycholanic Acid—7.48 gm. (0.02 mole) of 3(α)-hydroxy-Δ^{11}-cholenic acid in 60 cc. of dry chloroform were treated at 0° with 65 cc. of 0.7 N perbenzoic acid in chloroform. After 48 hours utilization of the perbenzoic acid was 94 per cent of 1 mole. The chloroform solution was shaken with water which contained 2 gm. of sodium bisulfite. The chloroform was removed under reduced pressure and the residue was washed with water and dissolved in 50 cc. of ethanol. The addition of 200 cc. of water caused the separation of 7.15 gm. of crystalline material, which was recrystallized from benzene. The melting point was 166–166.5° and $[α]_D = +39° \pm 2°$ (1 per cent in ethanol) (10).

Analysis—$C_{24}H_{38}O_4$. Calculated, C 73.78, H 9.81; found, C 73.77, H 9.71

Reduction of 3(α)-Hydroxy-11,12-epoxycholanic Acid and Methyl Ester (10)—2.02 gm. (0.005 mole) of methyl 3(α)-hydroxy-11,12-epoxycholanate were reduced in 25 cc. of acetic acid with 200 mg. of Adams' platinum oxide catalyst in the presence of 0.50 cc. of 1 N hydrochloric acid. After 48 hours slightly more than 1 mole of hydrogen had been absorbed. The platinum was removed and the residue after evaporation of the acetic acid under reduced pressure was hydrolyzed with sodium hydroxide in ethanol. The alcohol was removed and the alkaline solution was poured into hot dilute

aqueous hydrochloric acid. The precipitated acid, filtered, washed, and dried, was dissolved in 5 cc. of acetic acid by warming. About 1 gm. of material separated in crystalline form after the solution had been cooled to 5°. This was combined with the corresponding fraction from another 2.02 gm. sample of the starting material which had been reduced as described. The combined fractions (2.07 gm.) were suspended in boiling water to remove acetic acid and were dissolved in 20 cc. of 75 per cent acetone with the aid of heat. When cooled to 5°, crystals (1.17 gm.) separated. Two crystallizations from 6 cc. portions of hot acetic acid which were subsequently cooled to 5° gave 1.04 gm. of compound. This was precipitated twice from alkaline aqueous solution by addition to hot dilute hydrochloric acid. The partially purified acid was again crystallized from 7 cc. of 75 per cent acetone, heated to effect solution, and subsequently cooled to 5°. 831 mg. thus obtained were esterified in ether with diazomethane. The ester was dissolved in 3 cc. of benzene and was benzoylated by the addition of 1 cc. of pyridine and 1 cc. of benzoyl chloride. The flask was allowed to remain at room temperature overnight. Benzene was added, the solution was washed with water, and the benzene was removed under reduced pressure. 2 cc. of pyridine and 1 cc. of benzoyl chloride were added and the flask again remained overnight at room temperature. The residue was dissolved in benzene, washed free of pyridine with dilute hydrochloric acid and water, and the organic solvent was removed under reduced pressure. 701 mg. of material were separated in crystalline form after the residue was dissolved in methanol. Recrystallization from 20 cc. of boiling methanol yielded 613 mg. of rather long prisms which melted at 143–144° with a transition point at 131–132°. An authentic sample of methyl 3,12-dibenzoyldesoxycholate melted at 144–145° with a transition point at 129–130°. A mixture of the two samples melted at 144–144.5°. $[\alpha]_D = +121° \pm 2°$ (31.2 mg. in 6 cc. of methanol, 2 dm. tube). $[\alpha]_D$ of the authentic sample $= +122° \pm 2°$ (0.8 per cent in methanol).

780 mg. (0.002 mole) of $3(\alpha)$-hydroxy-11,12-epoxycholanic acid were reduced in 10 cc. of acetic acid with 100 mg. of platinum oxide catalyst in the presence of 1 drop of 1 N hydrochloric acid. After 23 hours about 75 per cent of 1 mole of hydrogen had been absorbed. Crystalline material which had separated was dissolved with the aid of heat, the platinum was removed by filtration, and the solution was concentrated under reduced pressure to 5 cc. 302 mg. of compound separated and were oxidized at 15° in acetic acid with 120 per cent of the theoretical amount of chromic acid. Addition of water precipitated the oxidized product which, after crystallization from 50 per cent ethanol, melted at 186–187° (13). The melting point was not depressed when the material was mixed with an authentic sample of dehydrodesoxycholic acid.

SUMMARY

For the introduction of oxygen in position 11 of the steroid nucleus $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid was prepared from desoxycholic acid through the pyrolysis of methyl $3(\alpha)$-hydroxy-12-benzoxycholanate. The preparation of methyl $3(\alpha)$-benzoxy-12-hydroxycholanate, $3(\alpha)$-hydroxy-12-benzoxycholanic acid, methyl 3,12-dibenzoyldesoxycholate, and $3(\alpha)$-hydroxy-11,12-epoxycholanate is described.

Analyses for carbon and hydrogen were made through the courtesy of Dr. Randolph T. Major in the laboratory of Merck and Company, Inc.

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STEROIDS DERIVED FROM BILE ACIDS

II. 3(α)-HYDROXY-11,12-DIBROMOCHOLANIC ACID AND RELATED COMPOUNDS

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In Paper I of this series (1) the preparation of 3(α)-hydroxy-Δ^{11}-cholenic acid (I) was reported. The present communication describes the products obtained by bromination of I and related compounds.[1]

Bromination of I in chloroform gave 3(α)-hydroxy-11,12-dibromocholanic acid (IV) which was converted in two steps to methyl 3(α)-acetoxy-11,12-dibromocholanate (VI). When the bromination was carried out in acetic acid, the same dibromo acid (IV) was obtained. However, another product separated from the reaction mixture which was shown by conversion to VI with diazomethane to be 3(α)-acetoxy-11,12-dibromocholanic acid (VII). This acid was obtained in varying yields which depended on the condition of the experiment. The ease with which the 3(α)-hydroxyl group is acetylated in acid medium will be referred to in a subsequent paper. Addition of bromine to II gave the dibromo ester (III) identical with the methyl ester prepared from IV. Bromination of methyl 3(α)-acetoxy-Δ^{11}-cholenate (V) gave the corresponding dibromide (VI) which could be prepared either by acetylation of III or esterification of VII. In all cases only one of the possible isomeric dibromides could be isolated.

Oxidation of 3(α)-hydroxy-11,12-dibromocholanic acid (IV) with chromic acid gave 3-keto-11,12-dibromocholanic acid (VIII).

Two of the eight compounds reported in this paper, II and V, have been prepared by Press and Reichstein (2). Lardon and Reichstein (3) regarded the methyl ester of VIII as a probable member of a mixture of bromo compounds which were by-products of the preparation of methyl 3-keto-11-hydroxy-12-bromocholanate but the dibromo acid (VIII) was not separated in analytically pure form.

Ott and Reichstein (4) chromatographed the products obtained by treatment of methyl 3(α)-acetoxy-Δ^{11}-cholenate with hypobromous acid and secured a fraction which was shown to be identical with "freshly prepared"

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[1] The work described in this paper was completed during 1941 and 1942. The results have been withheld from publication in agreement with other workers in this field.

methyl $3(\alpha)$-acetoxy-11,12-dibromocholanate,[2] but they did not give the physical characteristics of the compound last mentioned.

<div align="center">EXPERIMENTAL[3]</div>

All melting points were determined with the Fisher-Johns apparatus. *Methyl $3(\alpha)$-Hydroxy-Δ^{11}-cholenate (II) from I*—3.9 gm. of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid (I) were dissolved in 40 cc. of 0.1 N methanolic hydrogen

chloride at room temperature. The acid was completely esterified in 50 minutes. 350 mg. of sodium bicarbonate in 40 cc. of water were added,

[2] In a paper by Press, Grandjean, and Reichstein (5) which described the preparation of methyl $3(\alpha)$-acetoxy-11,12-dibromocholanate there is a typographical error. According to their paper the starting material which was brominated was methyl $3(\beta)$-acetoxy-Δ^{11}-cholenate; it therefore may be assumed that the compound described as the $3(\alpha)$-acetoxy derivative was methyl $3(\beta)$-acetoxy-11,12-dibromocholanate.

[3] Some of the compounds described in this paper were analyzed in the laboratory of Merck and Company, Inc., Rahway, New Jersey; the remainder were analyzed by Mr. William Saschek in the Department of Biochemistry, Columbia University, New York.

and the solution was cooled in an ice bath. 3.86 gm. of crystals which melted at 105.5–105.8° were obtained. $[\alpha]_D = +46° \pm 1°$ ($c = 1.012$ in methanol). Press and Reichstein have prepared this ester (2).

C$_{25}$H$_{40}$O$_3$. Calculated, C 77.27, H 10.37; found, C 77.48, H 10.09

Methyl 3(α)-Hydroxy-11,12-dibromocholanate (III) from IV—200 mg. of 3(α)-hydroxy-11,12-dibromocholanic acid (IV) (m.p. 178–179°) were dissolved in 3 cc. of methanol and 2 cc. of 0.5 N methanolic hydrogen bromide were added. After 17 hours at room temperature, the solution was cooled to 0°. The material (194 mg.) which separated was filtered from solution and washed with cold methanol; m.p. 175–175.5° with effervescence.[4] $[\alpha]_D = +54° \pm 2°$ ($c = 0.48$ in chloroform).

C$_{25}$H$_{40}$O$_3$Br$_2$.   Calculated.   C 54.75, H 7.35, Br 29.15
                    Found.   " 54.86, " 7.18, " 29.03

3(α)-Hydroxy-11,12-dibromocholanic Acid (IV) from I—3.74 gm. (0.01 mole) of 3(α)-hydroxy-Δ^{11}-cholenic acid (I) were dissolved in 75 cc. of chloroform and cooled to 4°. 19.5 cc. of 1.06 N bromine in chloroform, cooled to 4°, were added in one portion and after 1 minute the chloroform solution was washed with 50 cc. of water which contained 1 gm. of sodium bisulfite and with two 100 cc. portions of water. The chloroform solution was dried with sodium sulfate and concentrated to 15 cc. under reduced pressure. Ethyl acetate was added, the solution was again concentrated, and 25 cc. of ethyl acetate were added. The flask was cooled in an ice bath. The crystals (2.9 gm.) which separated were filtered from the solution and washed with ice-cold ethyl acetate, m.p. 178–179° with effervescence.[4] $[\alpha]_D = +57° \pm 2°$ ($c = 1$ in chloroform).

C$_{24}$H$_{38}$O$_3$Br$_2$.   Calculated.   C 53.94, H 7.17, Br 29.91
                    Found.   " 53.97, " 7.05, " 29.77

When IV was crystallized from acetic acid, it separated combined with 1 mole of solvent. 105.7 mg. required 3.59 cc. of 0.1 N sodium hydroxide;

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[4] The five 11,12-dibromo derivatives described in this paper melt with effervescence which is caused by the loss of hydrogen bromide. The melting point therefore is a decomposition point which is dependent on conditions which do not affect more stable compounds. Three of these conditions are the rate of increase of temperature, the temperature of the apparatus when the crystals are placed on the glass disk, and the physical state of the crystals, *i.e.*, whether they are large or finely ground. To control the second variable, the melting point was determined by the method of Cortese and Bauman (6). The highest temperature during which the material was stable for 30 seconds was several degrees higher than the melting point taken at an increase of temperature of 2° or 3° per minute. All melting points recorded in this paper were determined by the standard method.

calculated amount for 1 mole of IV combined with 1 mole of acetic acid = 3.56 cc. of 0.1 N sodium hydroxide.

Methyl 3(α)-Acetoxy-Δ¹¹-cholenate (V) from II—3.88 gm. (0.01 mole) of methyl 3(α)-hydroxy-Δ¹¹-cholenate (II) were dissolved in 5 cc. of acetic anhydride and 5 cc. of pyridine and kept at about 25° for 44 hours. Addition of ice and water precipitated crystalline material which was dissolved in benzene. The benzene was washed with dilute sulfuric acid, with a solution of sodium carbonate, and with water and was dried with sodium sulfate. The benzene was removed under reduced pressure; the residue was dissolved in 75 cc. of methanol which was concentrated under reduced pressure to about 20 cc. and cooled in an ice bath. The crystals (3.8 gm.) which separated, filtered from solution and washed with cold methanol, melted at 116–117°. A second crop of 0.23 gm., which melted at 115–116°, was obtained by dilution of the mother liquor with water. The analytical sample, prepared by recrystallizing from methanol, melted at 118.5–119.5°. $[α]_D = +51° \pm 2°$·(31.4 mg. in 3 cc. of acetone).

$C_{27}H_{42}O_4$. Calculated, C 75.30, H 9.83; found, C 75.17, H 9.81

Press and Reichstein have prepared this compound (2).

Methyl 3(α)-Acetoxy-11,12-dibromocholanate (VI) from III—7.7 gm. of methyl 3(α)-hydroxy-Δ¹¹-cholenate (II) were treated with 40 cc. of N bromine in chloroform at 0° to give methyl 3(α)-hydroxy-11,12-dibromocholanate (III). The chloroform was removed under reduced pressure and 50 cc. of acetic anhydride were added to the residue. The flask was heated for 3 hours at 90°, and was then cooled. The crystals (8.5 gm.), filtered from solution and washed with acetic acid, melted at 190–192° with effervescence.[4] Recrystallization from ethyl acetate raised the melting point to 195–196°. $[α]_D = +65° \pm 2°$ (c = 0.5 in chloroform).

$C_{27}H_{42}O_4Br_2$. Calculated. C 54.92, H 7.17, Br 27.07
Found. " 54.80, " 7.12, " 26.95

VI from V—860 mg. (0.002 mole) of methyl 3(α)-acetoxy-Δ¹¹-cholenate (V) in 10 cc. of chloroform, cooled in an ice bath, were brominated with 5 cc. of N bromine in chloroform. After 15 minutes the excess bromine was reduced with a dilute solution of sodium sulfite and the chloroform was washed with water, dried with sodium sulfate, and concentrated under reduced pressure to about 2 cc. 10 cc. of ethyl acetate were added and the solution was concentrated under reduced pressure to a small volume and chilled in ice. 838 mg. of crystals which separated, filtered from solution and washed with cold ethyl acetate, melted at 187–190° with effervescence.[4] A second crop (187 mg.) melted at 179–181°. Recrystallization three times from ethyl acetate raised the melting point to 192.5–193.5°. When

the substance was mixed with a sample of VI from III the melting point was 192–194°. $[\alpha]_D = +66° \pm 2°$ (30.5 mg. in 3 cc. of chloroform).

VI from VII—500 mg. of 3(α)-acetoxy-11,12-dibromocholanic acid (VII), m.p. 220°, were esterified in ether with diazomethane in the presence of a small amount of methanol. The ether was washed with hydrochloric acid and with water, dried with sodium sulfate, and removed under reduced pressure. The residue, crystallized from ethyl acetate, melted at 194–195° with effervescence.[4] When the substance was mixed with a sample of VI from III the melting point was 191–194°. $[\alpha]_D = +64° \pm 1°$ (20 mg. in 4 cc. of chloroform).

3(α)-Acetoxy-11,12-dibromocholanic Acid (VII) from IV—1 gm. of 3(α)-hydroxy-11,12-dibromocholanic acid (IV), m.p. 178–179°, was dissolved in 50 cc. of glacial acetic acid and 50 cc. of chloroform to which 10 cc. of 4.5 N hydrogen bromide in glacial acetic acid were added. After 16 hours at room temperature the solution was concentrated under reduced pressure to about 25 cc. The crystals which separated, filtered from solution and washed with glacial acetic acid, weighed 0.92 gm. and melted at 214–215° with effervescence.[4] Concentration of the mother liquor under reduced pressure gave a second crop (0.11 gm.) which melted at 213–215.5°. $[\alpha]_D = +62° \pm 2°$ (20 mg. in 4 cc. of chloroform). Four preparations of VII have had melting points between 224–228°. Recrystallization lowered the melting points from 5° to 10°.

$C_{26}H_{40}O_4Br_2$. Calculated. C 54.17, H 6.99, Br 27.73
Found. " 54.07, " 6.86, " 27.73

VII from I—187 mg. of 3(α)-hydroxy-Δ^{11}-cholenic acid (I) were dissolved in 5 cc. of glacial acetic acid to which 5 cc. of N bromine in glacial acetic acid were added. After 16 hours at 25° the crystals which had separated (134 mg.), filtered from solution and washed with acetic acid, melted at 218–219° with effervescence.[4] When the material was mixed with a sample of VII from IV, the melting point was 218–219°. $[\alpha]_D = +65° \pm 2°$ (27.4 mg. in 3 cc. of chloroform).

3-Keto-11,12-dibromocholanic Acid (VIII) from IV—2.73 gm. (0.005 mole) of 3(α)-hydroxy-11,12-dibromocholanic acid (IV) were dissolved in 20 cc. of chloroform, cooled in ice, and an ice-cold solution of 1.33 gm. of chromic acid in 1.4 cc. of water and 20 cc. of acetic acid was added. After 1 hour at 25°, the chloroform phase was separated by the addition of 200 cc. of water and was washed with water and dried with sodium sulfate. The chloroform was removed under reduced pressure; the residue, dissolved in 20 cc. of acetic acid, separated as crystals after the addition of water. The crystals, filtered from solution, washed with 80 per cent ice-cold acetic

acid, and dried in air, weighed 1.63 gm. The melting point was 178–179° with effervescence.[4] $[\alpha]_D = +46° \pm 2°$ ($c = 1.08$ in chloroform).

$C_{24}H_{36}O_3Br_2$. Calculated. C 54.14, H 6.81, Br 30.02
Found. " 54.21, " 6.62, " 30.08

SUMMARY

The preparation and properties of 3(α)-hydroxy-11,12-dibromocholanic acid and closely related compounds from 3(α)-hydroxy-Δ^{11}-cholenic acid are described.

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STEROIDS DERIVED FROM BILE ACIDS

III. DERIVATIVES OF Δ⁹,¹¹-CHOLENIC ACID WITH SUBSTITUENTS AT C₃ AND C₁₂*

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It has been shown by Reichstein and his associates (1, 2) and independently by us (3) that treatment of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid, or its derivatives, with perbenzoic acid leads to the formation of an oxide which may be reduced catalytically to desoxycholic acid. In the hope of obtaining an oxide of opposite configuration which might on reduction yield a C_{11}-hydroxy compound, $3(\alpha)$-hydroxy-11,12-dibromocholanic acid (I) (4) was treated with dilute aqueous alkali. The desired oxide, however, was not obtained, but instead an isomeric compound which was identified by Sarett (5) as $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid (II). As a by-product from the treatment with alkali $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid (3) was also separated. The structure of II was indicated by the conversion of the methyl ester (III) to a diacetate (IV) with acetic anhydride and pyridine and by the oxidation of II to 3,12-diketo-$\Delta^{9,11}$-cholenic acid (XIV) with chromic acid (6, 7). Under carefully controlled conditions we have further been able to oxidize II to $3(\alpha)$-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (VIII) (6–8).

Halogen was also eliminated from 3-keto-11,12-dibromocholanic acid (VII) with alkali and, by analogy with the formation of II, we formulate the product as 3-keto-12-hydroxy-$\Delta^{9,11}$-cholenic acid (XIII). Oxidation of XIII with chromic acid gave 3,12-diketo-$\Delta^{9,11}$-cholenic acid (XIV).

Compound II with acetic acid in the presence of a small amount of sulfuric acid at room temperature was rapidly converted (3 minutes) into a monoacetate (V) which with diazomethane gave the ester (VI). The free hydroxyl group in VI was shown to be at C₃ by oxidation with chromic acid followed by hydrolysis to give a monoketo monohydroxy acid identical with XIII which was formed as already described by treatment of 3-keto-11,12-dibromocholanic acid (VII) with aqueous alkali.

Because of the possibility of an allylic rearrangement in Ring C the acetoxyl group of V could be at C₉ or C₁₂. The following evidence suggests

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that the acetoxyl group is at C_{12} and the double bond is C_9,C_{11}.   The mono-acetate (VI) was converted in acetic anhydride and pyridine to a diacetate which was identical with the diacetate (IV) prepared from methyl $3(\alpha)$, 12-dihydroxy-$\Delta^{9,11}$-cholenate (III) in acetic anhydride and pyridine.   Alkaline hydrolysis of both mono and diacetyl derivatives, VI and IV, regenerated II.

When II was esterified with methanol and a trace of mineral acid, a compound (XI) was formed which differed from the ester (III) obtained by treatment of II with diazomethane.   We assign to this ester the structure methyl $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate on the basis of the following evidence.   Alkaline hydrolysis of the ester gave an acid (X) which contained one methoxyl group as shown by a Zeisel determination.   Acetylation of the methyl ester (XI) with acetic anhydride and pyridine gave a monoacetate (IX), while oxidation with 2 equivalents of chromic acid gave a monoketone (XII), identical with the product obtained from 3-keto-12-hydroxy-$\Delta^{9,11}$-cholenic acid (XIII) by the action of methanol and hydrochloric acid.   It follows, therefore, that the hydroxyl group of XI is at C_3 and that the methoxyl group is at C_9 or C_{12}.   We suggest that the methoxyl group is at C_{12} and that the double bond is C_9,C_{11} but full discussion will be withheld for another paper.   The following observations are consistent with position C_{12} for the methoxyl group, although they do not furnish unequivocal evidence.   Acetic acid and a small amount of sulfuric acid rapidly changed XI into a monoacetate identical with VI.   Oxidation of XI with an excess of chromic acid gave a small amount of methyl 3,12-diketo-$\Delta^{9,11}$-cholenate (XV).   In methanolic sodium hydroxide the methyl ester of $3(\alpha)$-hydroxy-11,12-dibromocholanic acid (I) was converted into an acid which with diazomethane gave XI.   This is analogous to the preparation of II from I with alkali in aqueous medium.   The formation of an allyl ether in Ring A of the steroid nucleus from a dibromide by the use of alcoholic alkali has been observed by Wieland and his coworkers (8).

Two of the compounds, VIII and XIV, reported in this paper have been described by other investigators. $3(\alpha)$-Hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (VIII) has been prepared by Chakravorty and Wallis (9), by Seebeck and Reichstein (6), and by Lardon and Reichstein (7).   Methyl 3,12-diketo-$\Delta^{9,11}$-cholenate (XIV) has been prepared by Seebeck and Reichstein (6) and by Lardon and Reichstein (7).

Seebeck and Reichstein state that the melting point of XV was the same as that of methyl 3,12-diketocholanate and that, although the melting point of a mixture of these two compounds did not show a depression, the specific rotation of the unsaturated ester in acetone was 20° lower than that of the saturated ester.   We have confirmed this observation, and

have shown further that the specific rotation of XIV and XV is greatly affected by the solvent and the presence of traces of acid. We have also observed that the specific rotations of several samples of XIV and XV in chloroform do not agree among themselves, although the melting points in each group were within a close range.

In this paper all monosubstituents at C_3 are in the α position. The configuration of the substituents at C_{12} will be discussed in another paper.

EXPERIMENTAL[1]

All melting points were determined on the Fisher-Johns apparatus.

$3(\alpha),12$-Dihydroxy-$\Delta^{9,11}$-cholenic Acid (II) from I—42.0 gm. of $3(\alpha)$-hydroxy-11,12-dibromocholanic acid (I) (4), which contained 1 mole of acetic acid of crystallization, were heated at 97° for 5 hours with 1000 cc. of 0.33 N sodium hydroxide. The solution was cooled, 200 cc. of benzene were added, and the solution was acidified with 200 cc. of normal hydrochloric acid. At the interface between the benzene and water a thick mass of crystals separated. The crystals were filtered, washed with water and benzene, and purified by solution in dilute sodium hydroxide and precipitation as described. 21.9 gm. of crystals were obtained which melted at about 150° with effervescence, resolidified, and melted again at 193–194°. When II was crystallized from acetone, the melting point was 198–199°. $[\alpha]_D =$ $+103° \pm 2°$ (30.1 mg. in 3 cc. of methanol). The sample for analysis was dried at 150° for 1 hour in a high vacuum.

$C_{24}H_{38}O_4$. Calculated, C 73.80, H 9.81; found, C 74.09, H 9.75

When II is crystallized from acetone, solvent is not retained in the crystals; when crystallized from benzene, 0.5 mole of benzene of crystallization is held firmly. Benzene of crystallization is lost and the crystals melt at about 150°. The melted material resolidifies and melts about 40° higher. 158 mg. of II crystallized from benzene lost 4.36 per cent in weight when heated at 128° for 15 hours under a vacuum. After heating the same material at 178° under a vacuum for 85 minutes longer, the loss in weight was 4.91 per cent. Total loss, 9.27 per cent; calculated loss for 0.5 mole of benzene, 9.09 per cent.

$3(\alpha)$-Hydroxy-Δ^{11}-cholenic Acid (3) from I—The benzene phase of the filtrates from the preparation of II was concentrated under reduced pressure and the residue was dissolved in normal sodium hydroxide through the aid of a little ethanol which was removed subsequently under reduced pressure. A white crystalline precipitate continued to separate for 3 days.

The solution was centrifuged and the precipitate was dissolved in alcohol; water was added and the solution was acidified with hydrochloric acid. $3(\alpha)$-Hydroxy-Δ^{11}-cholenic acid separated and was purified by crystalliza-

tion from benzene. The maximal yield was 25 per cent of the theoretically possible, based on the weight of the starting material. The acid added 1 mole of bromine. 200 mg. of the acid were reduced in 5 cc. of acetic acid

with hydrogen in the presence of 25 mg. of platinum oxide. In 15 minutes 11.6 cc. of hydrogen were absorbed (calculated, 11.4 cc.). The melting point of the lithocholic acid formed was 187–188°. The melting point of the $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid was 162–162.5° and when the substance was mixed with an authentic specimen there was no depression in the melting point. $[\alpha]_D = +45° \pm 2°$ (31.0 mg. in 3 cc. of ethanol).

II from III—100 mg. of methyl $3(\alpha)$,12-dihydroxy-$\Delta^{9,11}$-cholenate (III) were dissolved in 2.4 cc. of methanol to which was added 0.6 cc. of 5 N sodium hydroxide solution. The solution was kept at 50° for 46 hours, 50 cc. of water were added, and the methanol was removed under reduced pressure. The volume was made to 100 cc. with water, 10 cc. of benzene were added, and the solution was acidified with dilute acetic acid. 95 mg. of crystals, filtered from solution and washed with benzene and water, melted at 148–149°, resolidified, and melted at 189–192°. When the product was recrystallized from acetone and placed on the block previously heated to 190°, the melting point was 198–199°; when mixed with an authentic sample of II the melting point was 199–200°. $[\alpha]_D = +103° \pm 2°$ (31.2 mg. in 3 cc. of methanol).

II from IV—100 mg. of methyl $3(\alpha)$,12-diacetoxy-$\Delta^{9,11}$-cholenate (IV) were hydrolyzed as described in the previous paragraph. The product, 78 mg., melted with loss of solvent at 148–149°, resolidified, and melted at 183–186°. After recrystallization from acetone the melting point was 193.5–195° when the block was previously heated to 190°. When the crystals were mixed with an authentic sample of II, the melting point was 195–199°. $[\alpha]_D = +103° \pm 2°$ (30.2 mg. in 3 cc. of methanol).

II from V—To 109 mg. of $3(\alpha)$-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenic acid (V) in 9 cc. of methanol 0.6 cc. of 18 N sodium hydroxide was added and the solution was maintained at 60–70° for 3 hours. The solution was diluted with about 30 cc. of water and the methanol was removed under reduced pressure. About 5 cc. of benzene were added and the solution was acidified with hydrochloric acid. Crystals formed at the interface. The product melted at 150–160°, resolidified, and again melted at 193–195°. No depression of the melting point was observed when this product was mixed with an authentic sample of II. The yield was 86 mg.; after recrystallization from acetone, $[\alpha]_D = +104° \pm 2°$ (31.0 mg. in 3 cc. of methanol).

II from VI—3 gm. of methyl $3(\alpha)$-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenate (VI) were dissolved in 40 cc. of methanol to which 2.5 cc. of 18 N sodium hydroxide were added. The solution was allowed to stand at 50° for 20 hours. 300 cc. of water were added to the flask and the methanol was removed under reduced pressure to a volume of about 200 cc.; 100 cc. of benzene were added and the sodium hydroxide was neutralized to Congo

red with dilute hydrochloric acid. From the benzene-water suspension 2.61 gm. of crystals were filtered and washed with water and benzene. After reprecipitation from dilute alkaline solution by the addition of hydrochloric acid in the presence of benzene the crystals melted at between 145–150°, solidified, and melted at 196.5–198°. The product crystallized from acetone and dried at 100° melted at 191–195°; when mixed with a sample of II from I the melting point was 195–198°. $[\alpha]_D = \cdot +104° \pm 2°$ (30.0 mg. in 3 cc. of methanol).

Methyl 3(α),12-Dihydroxy-$\Delta^{9,11}$-cholenate (III) from II—500 mg. of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid (II) suspended in 50 cc. of ether and 5 cc. of methanol were esterified with diazomethane in ether. The solvents were removed under reduced pressure; the residue was dissolved in ether and the solution was washed with dilute sodium carbonate, dried with sodium sulfate, and evaporated to a small volume. Addition of petroleum ether caused the separation of crystals which melted at 87–88°. Recrystallization from methanol by the addition of water raised the melting point to 93–94°. When the crystals were placed on the block previously heated to 95°, the melting point was 97–97.5°. $[\alpha]_D = +98° \pm 2°$ (30.2 mg. in 3 cc. of methanol).

Methyl 3(α),12-Diacetoxy-$\Delta^{9,11}$-cholenate (IV) from VI—2 gm. of methyl 3(α)-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenate (VI) were dissolved in 4 cc. of pyridine and 4 cc. of acetic anhydride. After 21 hours the acetic anhydride was decomposed by addition of ice and the product was extracted with benzene. The organic phase was separated and washed successively with water, dilute sulfuric acid, sodium carbonate, and water; it was then dried with sodium sulfate. The benzene was removed under reduced pressure and the residue was crystallized from a small volume of ice-cold ligroin. The product weighed 1.12 gm. and melted at 80–81°. The analytical sample was prepared by several recrystallizations from petroleum ether and dilute methanol, m.p. 84.5–85°. $[\alpha]_D = +200° \pm 2°$ (31.2 mg. in 3 cc. of chloroform).

$$C_{29}H_{44}O_6. \text{ Calculated, C 71.27, H 9.08; found, C 71.53, H 9.08}$$

IV from III—100 mg. of methyl 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenate (III) were dissolved in 5 cc. of acetic anhydride and 5 cc. of pyridine and allowed to stand at room temperature for 19 hours. The acetic anhydride was decomposed with ice and the insoluble material was dissolved in benzene. The solution was washed with dilute sulfuric acid, with sodium bicarbonate solution, and with two portions of water. It was dried with sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in methanol, water was added to the point of turbidity, the solution was cooled in an ice-salt bath, and the product which separated

was filtered and washed with ice-cold 50 per cent methanol. Recrystallization from methanol-water gave 70 mg. of crystals which melted at 82–82.5°. When the product was mixed with a sample of IV from VI, the melting point was 82.5–84.5°. $[\alpha]_D = +198° \pm 2°$ (30.4 mg. in 3 cc. of chloroform).

3(α)-Hydroxy-12-acetoxy-Δ^{9,11}-cholenic Acid (V) from II—500 mg. of $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid (II) were dissolved in 50 cc. of glacial acetic acid to which 0.65 cc. of 5 N sulfuric acid was added. After 8 minutes the solution was mixed with 200 cc. of chloroform and washed with seven portions of water. The chloroform solution was evaporated to dryness under reduced pressure, and the residue was crystallized from ether. The product weighed 283 mg. and melted at 179–180°. After several recrystallizations from dilute methanol, the melting point was 185–186°. $[\alpha]_D = +206° \pm 2°$ (34.8 mg. in 3 cc. of methanol); $[\alpha]_D = +198° \pm 2°$ (25.0 mg. in 4 cc. of chloroform).

$C_{26}H_{40}O_5$. Calculated, C 72.18, H 9.32; found, C 72.04, H 9.59

Methyl 3(α)-Hydroxy-12-acetoxy-Δ^{9,11}-cholenate (VI) from V—1.500 gm. of $3(\alpha)$-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenic acid (V) were esterified in ether with diazomethane. The ether was concentrated under reduced pressure to about 2 cc., diluted with ligroin, and cooled in ice. The first crop of crystals, 0.742 gm., melted at 105–106°, and the second, 0.419 gm., at 107–108°. The analytical sample, prepared by recrystallization from ligroin, melted at 107.5–108°. $[\alpha]_D = +196° \pm 2°$ (30.6 mg. in 3 cc. of methanol).

$C_{27}H_{42}O_5$. Calculated. C 72.60, H 9.47, CH_3CO 9.63
Found. " 72.73, " 9.40, " 10.06

VI from XI—16.7 gm. (0.0400 mole) of methyl $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate (XI) were dissolved in 400 cc. of glacial acetic acid and 13 cc. of 5 N aqueous sulfuric acid were added. The temperature was 26°. At the end of 10 minutes the solution was poured into 600 cc. of benzene which was then washed with water, with dilute sodium bicarbonate solution, and with water. The benzene was dried over sodium sulfate and concentrated under reduced pressure to about 20 cc. 150 cc. of ligroin were added and the flask was cooled in an ice bath. The crystals, filtered from solution and washed with cold ligroin, weighed 13.4 gm. and melted at 107–107.5°. When the product was mixed with the analytical sample of VI from V, the melting point was 106–108°. $[\alpha]_D = +195° \pm 2°$ (28.9 mg. in 3 cc. of methanol).

3(α)-Hydroxy-12-keto-Δ^{9,11}-cholenic Acid (VIII) from II—1 gm. (0.00256 mole) of $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid (II) was dissolved in 50 cc. of acetic acid and 4 cc. of water. The flask was cooled in an ice bath and 2.7 cc. of 1.88 N chromic acid (2 equivalents) diluted to 20 cc. with acetic

acid were added dropwise over a period of 70 minutes to the solution stirred
mechanically. After 2 hours at room temperature the acetic acid, was
removed under reduced pressure and the residue was dissolved in 100 cc.
of 0.2 N sodium hydroxide. The alkaline solution was poured into 300 cc.
of boiling water which contained 20 cc. of N hydrochloric acid. The granu-
lar precipitate was separated by filtration and dissolved in 3 cc. of acetone,
2 cc. of water were added, and the solution was cooled in an ice bath. The
product which separated was filtered from solution and washed with ice-
cold 50 per cent acetone. The product, crystallized from acetone and water
five times, melted at 177–177.5°. $[\alpha]_D = +114° \pm 2°$ (33.3 mg. in 3 cc.
of methanol). When the crystals were mixed with an authentic sample of
$3(\alpha)$-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (VIII), m.p. 177.5–178°, the melt-
ing point of the mixture was 177–177.5°. $[\alpha]_D = +113° \pm 2°$ (33.5 mg.
of the authentic sample in 3 cc. of methanol) (6–8). Selective oxidation
of the hydroxyl group at C_{12} of desoxycholic acid has been reported (10).

Methyl $3(\alpha)$-Acetoxy-12-methoxy-$\Delta^{9,11}$-cholenate (IX) from XI—4.18 gm.
(0.01 mole) of methyl $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate (XI) were
dissolved in 10 cc. of acetic anhydride and 10 cc. of pyridine at room tem-
perature. After 44 hours chipped ice and about 100 cc. of water were
added; the crystals which separated were filtered and dissolved in benzene.
The benzene solution was washed with dilute sulfuric acid, with a solution
of sodium bicarbonate, and with water, dried with sodium sulfate, and con-
centrated under reduced pressure. The last traces of benzene were re-
moved by the addition of 20 cc. of methanol and concentration under
reduced pressure. The residue was dissolved in about 15 cc. of methanol,
and the solution was cooled in ice and diluted with cold 75 per cent meth-
anol. The product, filtered from solution and washed with 75 per cent
methanol, weighed 4.12 gm. and melted at 100.5–101.5°. Recrystalliza-
tion from methanol gave a product which melted at 102–103°. $[\alpha]_D = +140° \pm 2°$ (29.8 mg. in 3 cc. of chloroform).

$C_{28}H_{44}O_4$. Calculated, C 73.00, H 9.63; found, C 72.80, H 9.36

$3(\alpha)$-Hydroxy-12-methoxy-$\Delta^{9,11}$-cholenic Acid (X) from XI—5 gm. of
methyl $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate (XI) were dissolved in
200 cc. of warm methanol. The flask was cooled to 15°, 40 cc. of normal
sodium hydroxide solution were added, and the solution was allowed to
remain at room temperature overnight. The solution was concentrated
to about 25 cc. under reduced pressure, diluted with 400 cc. of water, and
concentrated to 350 cc. to remove the last traces of methanol. 200 cc. of
benzene and 40 cc. of normal hydrochloric acid were added with stirring;
the benzene phase was separated, washed with water, dried with sodium
sulfate, and evaporated to about 25 cc. Crystals which separated after

addition of 10 cc. of ligroin were filtered, washed with ligroin, dissolved in 60 cc. of ethyl acetate, and recrystallized by addition of 105 cc. of ligroin at 0°. The product, 4.5 gm., melted at 147–148°. Recrystallization from 10 cc. of acetone by the addition of 4 cc. of water raised the melting point to 148.5–149°. $[\alpha]_D = +138° \pm 2°$ ($c = 0.51$ in chloroform).

$$C_{25}H_{40}O_4. \quad \text{Calculated.} \quad \text{C } 74.22, \text{ H } 9.96, \text{ CH}_3\text{O } 7.67$$
$$\text{Found.} \quad \text{“ } 74.22, \text{ “ } 9.75, \text{ “ } 7.73$$

Methyl 3(α)-Hydroxy-12-methoxy-Δ⁹,¹¹-cholenate (XI) from II—10.2 gm. of $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenic acid (II) were dissolved in 100 cc. of methanol and 0.2 cc. of concentrated hydrochloric acid was added. After 16 hours at room temperature large transparent crystals had separated. After concentration of the solution a total of 9.2 gm. was obtained. After recrystallization from methanol the melting point was 160–161°. $[\alpha]_D = +132° \pm 2°$ (30.0 mg. in 3 cc. of chloroform); $[\alpha]_D = +130° \pm 2°$ (30.9 mg. in 3 cc. of methanol).

$$C_{26}H_{42}O_4. \quad \text{Calculated, C } 74.59, \text{ H } 10.11; \text{ found, C } 74.43, \text{ H } 10.05$$

XI from III—100 mg. of methyl $3(\alpha),12$-dihydroxy-$\Delta^{9,11}$-cholenate (III) were dissolved in 10 cc. of methanol at room temperature, and 2 drops of concentrated hydrochloric acid were added. After 18 hours the methanol was concentrated to about 1 cc. under reduced pressure and the crystals which separated were recrystallized from methanol. The melting point was 160–161° and a mixture of the crystals with a sample of XI from II melted at 160–161°. $[\alpha]_D = +130° \pm 2°$ ($c = 1$ in methanol).

XI from X—875 mg. of $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenic acid (X) were esterified in 50 cc. of ether with diazomethane. The ether solution was washed with dilute hydrochloric acid and water, dried with sodium sulfate, and concentrated to dryness under reduced pressure. The residue was crystallized from methanol and gave a product which melted at 158–160°; when mixed with a sample of XI from II, the melting point was 159–160°. $[\alpha]_D = +130° \pm 2°$ ($c = 1$ in methanol).

XI from IV—100 mg. of methyl $3(\alpha),12$-diacetoxy-$\Delta^{9,11}$-cholenate (IV) were suspended in 2 cc. of a methanolic solution which contained hydrochloric acid.[2] The flask was allowed to remain at 17° for 5 days. During this interval the starting material dissolved and crystals separated. The crystals, filtered from solution and washed with ice-cold dilute methanol, weighed 65 mg. and melted at 157.5–158.5°. After recrystallization from

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[2] The solution was 84 per cent methanol, 16 per cent water, and contained 1 N hydrochloric acid. It was prepared by the addition of 1.67 cc. of 12 N aqueous hydrochloric acid and 1.95 cc. of water to 16.8 cc. of methanol. The reasons for the use of this solution will be given in a later communication.

2 cc. of 85 per cent methanol, the melting point was 160–161°, and showed
no depression on mixture with an authentic sample of XI. $[\alpha]_D$ =
+132° ± 2° (31.2 mg. in 3 cc. of chloroform).

XI from VI—446 mg. of methyl 3(α)-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenate
(VI) were dissolved with agitation in 2 cc. of a methanolic acid solution
described in the preceding paragraph.   After 72 hours at 17°, 382 mg. of
material which separated were filtered from solution and washed with cold
75 per cent methanol.   After recrystallization from methanol the melting
point was 159–160°.   The melting point of a mixture with an authentic
sample of XI was 161–162°. $[\alpha]_D$ = +133° ± 2° (30.8 mg. in 3 cc. of
chloroform).

XI from IX—460 mg. of methyl 3(α)-acetoxy-12-methoxy-$\Delta^{9,11}$-cholenate
(IX) were added to 18 cc. of a methanolic acid solution as in the preceding
paragraph.   The flask was allowed to remain at 17° for 6 days, during
which time crystals separated.   The product, 296 mg., melted at 158–160°;
after recrystallization from 3 cc. of 85 per cent methanol, the melting point
was 160–161°.   When the product was mixed with an authentic sample of
XI, the melting point was 161–162°. $[\alpha]_D$ = +132° ± 2° (30.1 mg. in
3 cc. of chloroform).

XI from Methyl Ester of I—11.0 gm. (0.0200 mole) of methyl 3(α)-
hydroxy-11,12-dibromocholanate (4) were refluxed for 6 hours in 800 cc.
of methanol which contained 4 gm. of sodium hydroxide.   The methanol
was removed under reduced pressure and the residue was dissolved in 200 cc.
of water.   200 cc. of benzene were added to the flask and the sodium salt
was acidified with acetic acid and shaken.   The benzene was washed with
water, dried with sodium sulfate, and removed under reduced pressure.
The oily residue was esterified with diazomethane in ether.   Crystals
separated from the ether on evaporation.   After recrystallization from
ether, 593 mg. of the product melting at 160–161° were obtained which
did not depress the melting point of XI (161–163°). $[\alpha]_D$ = +131° ±
1° (40 mg. in 4 cc. of chloroform).

Methyl 3-Keto-12-methoxy-$\Delta^{9,11}$-cholenate (XII) from XI—1.2 gm. (0.003
mole) of methyl 3(α)-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate (XI) were dis-
solved in 5 cc. of chloroform and 10 cc. of acetic acid and chilled in ice
water.   12 cc. of 2 N chromic acid in acetic acid (8 equivalents) were added
and the solution was kept in the ice bath 30 minutes and at 25° for 5 hours.
Water was added and the aqueous phase was extracted with chloroform.
Titration of the aqueous phase indicated the reduction of 4.7 equivalents
of chromic acid.   The chloroform solution was washed with water, dried
with sodium sulfate, and evaporated under reduced pressure.   The residue
was dissolved in 150 cc. of a mixture of petroleum ether and benzene in the
ratio of 1:2.   The solution was passed through a column of 40 gm. of

aluminum oxide and the material was eluted with twelve 400 cc. portions of petroleum ether and benzene (1:2). The eluted material, 623 mg., was crystallized from methanol and water and then from ligroin. 415 mg. of crystalline product which melted at 98–99° were separated. $[\alpha]_D = +110° \pm 1°$ (45.7 mg. in 5 cc. of chloroform).

$C_{24}H_{40}O_4$. Calculated, C 74.96, H 9.68; found, C 74.75, H 9.50

XII from XIII—200 mg. of 3-keto-12-hydroxy-$\Delta^{9,11}$-cholenic acid (XIII), m.p. 190–191°, were dissolved in 20 cc. of methanol and 0.28 cc. of concentrated hydrochloric acid was added. After 4.5 hours at room temperature the methanolic solution was diluted with water until crystals separated. After two recrystallizations from methanol and water, 100 mg. of a product were obtained which melted at 99.5–100°, and when mixed with the oxidation product of XI with chromic acid, the melting point was 98.5–99.5°. $[\alpha]_D = +112° \pm 2°$ (33.7 mg. in 3 cc. of chloroform).

3-Keto-12-hydroxy-$\Delta^{9,11}$-cholenic Acid (XIII) from VI—3.35 gm. (0.0075 mole) of methyl 3(α)-hydroxy-12-acetoxy-$\Delta^{9,11}$-cholenate (VI) were dissolved in 90 cc. of glacial acetic acid and cooled to 15°. 23.9 cc. of 1.88 N chromic acid in acetic acid were added slowly, and the temperature was maintained between 13–15° for 30 minutes. The solution was poured into a flask which contained 200 cc. of benzene and 500 cc. of water. The benzene was separated and the aqueous phase was extracted with 50 cc. of benzene. The benzene solutions were combined and washed with water, a dilute sodium bicarbonate solution, again with water, and were dried with sodium sulfate and evaporated under reduced pressure. The residue was dissolved in 40 cc. of methanol to which were added 2.5 cc. of 18 N aqueous sodium hydroxide. After 16 hours at 25° and 20 hours at 50°, 200 cc. of water were added and the methanol was removed under reduced pressure. The solution was made to 200 cc. with water, 100 cc. of benzene were added, and the solution was acidified with a slight excess of acetic acid. The crystals, 2.75 gm., which separated from the benzene melted at 186–187°, and after recrystallization from acetone-water, the melting point was 190.5–191.5°. $[\alpha]_D = +71° \pm 2°$ (31.7 mg. in 3 cc. of chloroform).

$C_{24}H_{36}O_4$. Calculated, C 74.19, H 9.34; found, C 73.97, H 9.20

XIII from VII—5.34 gm. (0.01 mole) of 3-keto-11,12-dibromocholanic acid (VII) were dissolved in 100 cc. of 95 per cent ethanol and 100 cc. of water which contained 3.36 gm. of sodium bicarbonate. After 20 hours at 55° the alcohol was removed under reduced pressure; 200 cc. of benzene were added and the mixture was acidified with 7 cc. of 50 per cent acetic acid. The benzene was washed three times with 200 cc. portions of water and concentrated under reduced pressure. Crystals separated when the

volume was about 40 cc.   The product weighed 1.6 gm. and after recrystallization once from ethyl acetate and ligroin and twice from ethyl acetate effervesced at 115°, resolidified, and melted at 190.5–191.5°.   When the crystals were mixed with a sample of XIII from VI, the melting point was 190.5–191.5°. $[\alpha]_D = +73° \pm 1°$ (40 mg. in 4 cc. of chloroform).

3,12-Diketo-Δ⁹,¹¹-cholenic Acid (XIV) from XV—100 mg. of methyl 3,12-diketo-Δ⁹,¹¹-cholenate (XV), m.p. 129.5–130.5°, were dissolved in 10 cc. of ethanol and 10 cc. of 0.1 N sodium hydroxide.   The solution was warmed for 1 hour at 50°.   12 cc. of 0.1 N sulfuric acid were added and the ethanol was removed under reduced pressure.   The crystals which separated,

TABLE I

Specific Rotation of Compounds XIV and XV

Compound	M.p.	Specific rotation in various solvents, $c = 1$					
		CHCl₃	CHCl₃ + 0.001 N HCl	CH₃OH	CH₃OH + 0.001 N HCl	Acetone	Acetone + 0.001 N HCl
	°C.						
XV	131 –132	77	94	87	106	74	75
Methyl 3,12-diketo-cholanate	131 –133	93	93	96	101	89	90
XIV, 3 samples	197 –198	67 ± 4					
	198 –199	72 ± 2					
	199 –200	82 ± 2					
XV, 3 "	130 –131	68 ± 2					
	129.5–130.5	64 ± 2					
	131 –132	77 ± 2					

filtered from solution and washed with water, melted at 193–195°.   When recrystallized twice from acetone and water and dried at 100° and 13 mm. pressure, the product melted at 197–198° $[\alpha]_D = +67° \pm 4°$ (18.7 mg. in 3 cc. of chloroform); see Table I.

$C_{24}H_{34}O_4$.   Calculated, C 74.57, H 8.86; found, C 74.59, H 9.02

XIV from XIII—100 mg. of 3-keto-12-hydroxy-Δ⁹,¹¹-cholenic acid (XIII) were dissolved in 9 cc. of acetic acid and 1 cc. of water.   The flask was cooled in an ice bath and 0.6 cc. of 1.88 N chromic acid in acetic acid was added.   After 10 minutes in the ice bath the flask was kept at room temperature for 1 hour, water was added, and the solution was extracted with ether.   The ether was washed with water, dried with sodium sulfate, and removed under reduced pressure.   The residue was crystallized from acetone by the addition of a small amount of water.   The melting point

was 196–198°. Recrystallization from acetone and water gave 63 mg. of a product which melted at 198–199°. When these crystals were mixed with a sample of XIV prepared from VIII, the melting point was 199–200°. $[\alpha]_D = +72° \pm 2°$ (29.7 mg. in 3 cc. of chloroform); see Table I.

XIV from VIII—776 mg. (0.002 mole) of 3(α)-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (VIII) were dissolved in 5 cc. of benzene and 20 cc. of acetic acid. The solution was cooled in an ice bath and 2.5 cc. of 2 N chromic acid were added. After 30 minutes in the ice bath the flask was kept at room temperature for 1.5 hours. Water and benzene were added; the benzene was washed with water, dried with sodium sulfate, and removed under reduced pressure. The residue, after crystallization from an acetone-water mixture, weighed 598 mg. The melting point was 199–200°. The melting point of the crystals mixed with a sample of XIV from II was 199–200°. $[\alpha]_D = +82° \pm 2°$ (30.5 mg. in 3 cc. of chloroform); see Table I.

Methyl 3,12-Diketo-$\Delta^{9,11}$-cholenate (XV) from II—The oxidation of 1.95 gm. of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid (II) with chromic acid was carried out in 20 cc. of glacial acetic acid and 20 cc. of 2 N chromic acid in acetic acid, at 12–15°. After 30 minutes 4.5 equivalents of chromic acid had been reduced. Water and benzene were added; the benzene was washed with water, dried over sodium sulfate, and removed under reduced pressure. The crude oxidation product was esterified with diazomethane and the methyl ester was subjected to chromatographic adsorption and elution from a column of 60 gm. of aluminum oxide. The portion eluted by benzene which contained 0.1 per cent methanol weighed 1.45 gm., and when recrystallized from ligroin (b.p. 70–90°) to constant melting point and dried in a vacuum at 100° for 2 hours, melted at 130–131°. $[\alpha]_D = +68° \pm 2°$ (38.2 mg. in 4 cc. of chloroform); see Table I.

$C_{25}H_{36}O_4$. Calculated, C 74.96, H 9.05; found, C 75.04, H 9.09

XV from XI—From the oxidation of 1.2 gm. of methyl 3(α)-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate (XI) (see XII from XI), 71 mg. of material were eluted from the column of aluminum oxide with benzene. After two recrystallizations from ligroin the melting point was 129.5–130.5°. $[\alpha]_D = +64° \pm 2°$ (20.3 mg. in 4 cc. of chloroform). When the crystals were mixed with the methyl ester of the product obtained by oxidation of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid (II), the melting point was 128.5–130.5°.

SUMMARY

The preparation and properties of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid and twelve closely related compounds with hydroxyl, acetoxyl, methoxyl, or ketone groups at C_3 and C_{12} are described.

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THE INTRODUCTION OF AN 11-HYDROXYL GROUP IN 12-KETOSTEROIDS*

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The most active hormones of the adrenal cortex, in their effect on carbohydrate metabolism, contain oxygen atoms attached at the 11 position of the steroid nucleus. A systematic study of the preparation of compounds containing a hydroxyl group in the 11 position has been made essentially according to the procedures reported (1, 2) for the preparation of 3,11-dihydroxy-12-ketocholanic acid. This was accomplished by brominating 3-acetoxy-12-ketocholanic acid and hydrolyzing the resulting monobromide. This method is similar to that used by Wieland and Posternak (3) and by Barnett and Reichstein (4) for the preparation of 11-hydroxy-12-ketocholanic acid.

$$R = —CH(CH_3)CH_2COOH, \quad —CH(CH_3)COOH, \quad \text{and} \quad —COOH$$

The method has been extended, in this investigation, to the preparation of the nor, bisnor, and etio homologues (IV) of 3,11-dihydroxy-12-keto-cholanic acid. In the case of the bisnor acid (IV, R = —CH(CH₃)COOH) the free acid did not crystallize readily and was characterized as its methyl ester which was compared to methyl 3-hydroxy-12-ketobisnorcholanate and found to be quite different.

Epimeric mixtures would be expected from this series of reactions. Their separation, as free acids, is very difficult. The methyl ester of the bisnor acid was, however, separated into two nicely crystalline fractions, one melting at 70–80° and giving a specific rotation of +67° ± 2° and the other melting at 146.5–147.5° and giving a specific rotation of +62° ± 1°. In the case of the etio acid fractional crystallization of the reaction product appeared to give two different compounds. The least soluble fractions had the lower rotations. When methanol was used as the solvent, two distinct crystalline forms were obtained which were long needles and short thick prisms. The fact that they could be readily changed from one form to the other indicated that they might be dimorphic forms of only one substance. The needles separated when concentrated solutions were rapidly cooled, and prisms when the solutions were allowed to cool slowly. Prisms were also obtained when dilute solutions were allowed to evaporate slowly.

EXPERIMENTAL[1]

3,11-Dihydroxy-12-ketonorcholanic Acid—A solution of 4 gm. of 3-hydroxy-12-ketonorcholanic acid (5) in 12 ml. of acetic acid and 2 ml. of acetic anhydride was refluxed for 1 hour. To the cooled solution were added 30 ml. of acetic acid and 5 ml. of acetic acid saturated with hydrogen bromide. With stirring at a temperature of 50–60° a solution of 0.6 ml. of bromine in 30 ml. of acetic acid was slowly added during a period of 2 hours. The stirring and warming were continued for about 4 hours more and then the solution was cooled and poured into 500 ml. of water. The 3-acetoxy-11-bromo-12-ketonorcholanic acid separated as a flocculent precipitate and was collected on a filter and dried. The yield was about 5 gm.

The crude bromo compound was dissolved in 100 ml. of 20 per cent methanolic potassium hydroxide and the solution was refluxed for 20 minutes. After being diluted to about 1 liter with water the solution was acidified and the precipitate was collected on a filter and dried. The yield was 3.8 gm. of an almost white amorphous powder. This crude 3,11-dihy-

droxy-12-ketonorcholanic acid could be easily crystallized from benzene containing a little methanol or from 50 per cent aqueous ethanol, but the crystals (m.p. 180–195°) were not pure. The mixture was separated by fractional crystallization from 50 per cent aqueous ethanol. The less soluble fraction proved to be unchanged 3-hydroxy-12-ketonorcholanic acid and the more soluble fraction the desired 3,11-dihydroxy-12-ketonorcholanic acid, m.p. 201–205°. The yield was small.

Owing to the unreactive nature of the keto group in 3,11-dihydroxy-12-ketonorcholanic acid a better method was developed for its purification. Most of the starting material and other impurities could be removed by conversion to their semicarbazones; the desired product, however, did not react under the conditions used. A mixture of the crude reaction product, 4 gm. of semicarbazide hydrochloride, 4 gm. of sodium acetate, 125 ml. of 95 per cent ethanol, and 40 ml. of water was refluxed for 2 hours. Part of the solvent was removed by distillation and the mixture was poured into 500 ml. of water. The precipitate was collected, dried, and extracted with ether in a Soxhlet extractor. Concentration of the ether solution gave material which after one crystallization from 50 per cent ethanol melted at about 200° and weighed 2.15 gm. (52 per cent based on 3-hydroxy-12-ketonorcholanic acid). A sample crystallized from benzene and twice from 50 per cent ethanol (decolorizing charcoal was used the second time) gave a product melting at 203–207°; $[\alpha]_D^{27} = +48° \pm 1°$ (40.1 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +1.01° \pm 0.01°$; l, 1 dm.).

Analysis—$C_{23}H_{36}O_5$. Calculated, C 70.37, H 9.25; found, C 70.37, H 9.08

Methyl 3,11-Dihydroxy-12-ketobisnorcholanate—By a method similar to that used for the nor acid a yield of 1.7 gm. of crude 3-acetoxy-11-bromo-12-ketobisnorcholanic acid was obtained from 1.33 gm. of 3-hydroxy-12-keto-bisnorcholanic acid (5). This crude bromo compound was hydrolyzed by methanolic potassium hydroxide in a manner similar to that described for the nor acid, giving 1.2 gm. of crude amorphous 3,11-dihydroxy-12-keto-bisnorcholanic acid which melted at about 205–210° after sintering at 115–130°.

This crude acid was esterified by dissolving it in 20 ml. of methanol, adding 1 ml. of acetyl chloride, refluxing for 15 minutes, and then allowing it to stand overnight. The solution was poured into ice water and extracted with ether. The ether solution was washed with 5 per cent sodium carbonate solution, then with water, and dried over sodium sulfate. The ether was removed and the residue crystallized from a mixture of methanol and water by allowing the solution to evaporate slowly. The first crop of needles (0.22 gm.) melted at 75–83° and after two more crystal-

lizations melted at about 70–80°; $[\alpha]_D^{27} = +67° \pm 2°$ (31.7 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +1.06° \pm 0.02°$; l, 1 dm.).

Analysis—$C_{23}H_{36}O_5$. Calculated, C 70.37, H 9.25; found, C 70.59, H 9.55

By concentrating the filtrate from the first fraction and fractionally crystallizing the product from aqueous methanol a sample (0.076 gm.) of nicely crystalline material melting at 146.5–147.5° was obtained; $[\alpha]_D^{27} = +62° \pm 1°$ (40.4 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +1.25° \pm 0.02°$; l, 1 dm.).

Analysis—$C_{23}H_{36}O_5$. Calculated, C 70.37, H 9.25; found, C 70.18, H 9.31

Methyl 3-Hydroxy-12-ketobisnorcholanate—A 0.5 gm. sample of 3-hydroxy-12-ketobisnorcholanic acid was methylated by a procedure similar to that described above. On concentration of the ether solution the ester separated in crystals melting at 164–166°. Crystallization from aqueous methanol and then from a mixture of ether and petroleum ether gave 0.231 gm. of ester melting at 165–167°.

Analysis—$C_{23}H_{36}O_4$. Calculated, C 73.36, H 9.64; found, C 73.48, H 9.41

3,11-Dihydroxy-12-ketoetiocholanic Acid—From 1.446 gm. of 3-hydroxy-12-ketoetiocholanic acid (5) were obtained 1.914 gm. of crude 3-acetoxy-11-bromo-12-ketoetiocholanic acid by a method similar to that used for the nor acid. This crude bromo acid was hydrolyzed by methanolic potassium hydroxide, as described above. The product crystallized from methanol, giving 0.74 gm. of material melting at 242–245°; $[\alpha]_D^{28} = +64° \pm 2°$ (28.6 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +0.92° \pm 0.02°$; l, 1 dm.). Concentration of the mother liquors gave an additional 0.2 gm. of crystalline material.

Fractional crystallization, from methanol, of this and the products from other runs gave many fractions, all of which had nearly the same melting points, but their rotations varied widely. One of the less soluble fractions (prisms) melted at 244–248°; $[\alpha]_D^{27} = +57.5° \pm 2°$ (21.2 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +0.61° \pm 0.01°$; l, 1 dm.).

Analysis—$C_{20}H_{30}O_5$. Calculated. C 68.55,   H 8.63
                        Found.   68.74, 69.01, " 8.89, 8.64

From the same run a more soluble fraction melted at 238–240° on the Fisher-Johns melting point block.

Analysis—Found, C 69.16, H 8.88

One of the fractions with a higher specific rotation melted at 242–250°; $[\alpha]_D^{27} = +87° \pm 2°$ (20.2 mg. made up to 2 ml. with 95 per cent ethanol, $\alpha = +0.88° \pm 0.02°$; l, 1 dm.). ·

SUMMARY

1. The nor, bisnor, and etio homologues of 3,11-dihydroxy-12-ketocholanic acid have been prepared from the corresponding 3-acetoxy-12-ketocholanic acids by first brominating and then hydrolyzing the bromo compounds.

2. These reactions should give epimeric mixtures which were separated in the bisnor series.

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THE STRUCTURE OF SOME DERIVATIVES OF 3(α)-HYDROXY-$\Delta^{9,11}$-CHOLENIC ACID

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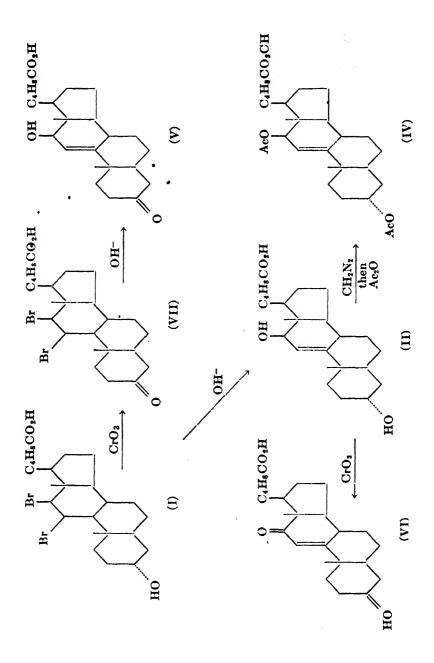
From the unsaturated steroid 3(α)-hydroxy-$\Delta^{9,11}$-cholenic acid Kendall and coworkers prepared two derivatives which appeared to be possible intermediate compounds in the partial synthesis of some of the hormones of the adrenal cortex (1). The first compound was an acid $C_{24}H_{38}O_4$ (II) obtained by treatment of 3(α)-hydroxy-11,12-dibromocholanic acid with dilute aqueous sodium hydroxide. The second derivative (III) was obtained by treatment of II with mineral acid in methanol. The work described in this paper was undertaken at the request of the above authors before the determination of the structure of these compounds had been completed. The results of this investigation indicate that the structure of II is 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid[1] and that III has the structure methyl 3(α)-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate.

The formulation II for the acid $C_{24}H_{38}O_4$ was based on the following reactions. The methyl ester of II gave a diacetate (IV) with pyridine and acetic anhydride. Alkaline hydrolysis of the diacetate regenerated the original dihydroxy acid. Mild oxidation of II with chromium trioxide gave a new acid of melting point 194° (VI). That the oxidation had affected only the C_3-hydroxyl group was shown not to be the case by oxidizing 3(α)-hydroxy-11,12-dibromocholanic acid (I) to the corresponding keto acid (VII) and treating the latter with dilute sodium hydroxide to give a hydroxy keto acid (V) different from VI. The absorption spectrum of VI, for the determination of which the author is indebted to Mr. W. A. Bastedo, Jr., of this laboratory, showed the presence of an α,β-unsaturated ketone group. Finally it was clearly shown to have the structure VI by its formation from oxidation of an authentic sample of 3(α)-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid, kindly supplied by Dr. Everett S. Wallis of Princeton University.

It was found by Kendall and coworkers (1) that the empirical formula of the product III obtained by treatment of II with mineral acid in methanol was $C_{26}H_{42}O_4$. They also showed that the compound was a methyl ester of an acid which contained a methoxyl group. A study of the action of acetic acid and perbenzoic acid on this compound supported this for-

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[1] It is of interest to note here the similarity between 3-hydroxy-11,12-dibromocholanic acid and 2,3-dibromocholanic acid in their reactions with alkali (2).

mulation and indicated that its structure was methyl 3(α)-hydroxy-12-methoxy-$\Delta^{9, 11}$-cholenate.

Acetic acid brought about cleavage of the methyl ether to give the acetate

CH$_3$O   C$_4$H$_8$CO$_2$CH$_3$

CH$_3$OH
H$^+$

HOAc
then NaOH

HO

(III)

OH   C$_4$H$_8$CO$_2$H

HO

(II)

ϕCO$_3$H

CH$_3$O   C$_4$H$_8$CO$_2$CH$_3$

O

HO

(VIII)

CrO$_3$

CH$_3$O   C$_4$H$_8$CO$_2$CH$_3$

O

O

(IX)

NH$_2$OH

CH$_3$O   C$_4$H$_8$CO$_2$CH

O

HON

(X)

of II. With III perbenzoic acid gave a hydroxy methoxy oxide (VIII) which was stable toward acetic anhydride at 100° and relatively stable toward mineral acids. This oxide readily gave a keto methoxy oxide (IX), thence a methoxy oxide oxime (X).

In order to study the effect of oxidizing agents on Ring C in III, it seemed

desirable at an early stage in the work to convert III to the 3-desoxy acid (XIII). This was accomplished by conversion of III to the tosylate (XI), refluxing the latter with collidine, and hydrolysis to the corresponding unsaturated acid (XII), followed by hydrogenation[2] to 12-methoxy-$\Delta^{9, 11}$-cholenic acid (XIII) (cf. (3)). The latter was converted to the methyl ester

CH_3O $C_4H_8CO_2CH_3$

III ϕSO_2Cl $\dfrac{\text{Collidine}}{\text{then NaOH}}$

ϕSO_2

(XI)

CH_3O $C_4H_8CO_2H$ CH_3O $C_4H_8CO_2H$

$\xrightarrow{H_2, \text{ Pt}}$ $\xrightarrow[\text{then } \phi CO_3H]{CH_2N_2}$

(XII)   (XIII)

CH_3O $C_4H_8CO_2CH_3$

O

(XIV)

and treated with perbenzoic acid. It reacted at a moderate rate to give the crystalline oxide (XIV).

No significant products could be isolated from the action of other oxidizing agents on XIII. It was found that XIII was stable to alcoholic selenium dioxide and to osmium tetraoxide. Bromine in acetic acid reacted over a period of 10 minutes to give a crystalline product containing ap-

[2] Carried out by Dr. Ralph Mozingo of this laboratory.

proximately 1 atom of bromine. Chromic acid and potassium permanganate reacted slowly; the products could not be obtained pure.

The author wishes to express his appreciation to Dr. Randolph T. Major and to Dr. Karl Folkers for their active interest in this work. To Dr. Everett S. Wallis of Princeton University is due the author's appreciation for his stimulating suggestions and close association with this work.

<div align="center">EXPERIMENTAL[3,4]</div>

*3(α)-Hydroxy-11,12-dibromocholanic Acid (I), 3(α),12-Dihydroxy-Δ[9,11]-cholenic Acid (II), Methyl 3(α)-Hydroxy-12-methoxy-Δ[9,11]-cholenate (III)—*The details for the preparation of these compounds are contained in two papers by Kendall and coworkers (1, 4).

*3,12-Diketo-Δ[9,11]-cholenic Acid (VI); (a) from II—*To a solution of 500 mg. of 3,12-dihydroxy-Δ[9,11]-cholenic acid-ether complex in 40 cc. of glacial acetic acid a solution of 500 mg. of chromium trioxide in 20 cc. of 50 per cent acetic acid was added dropwise with stirring at 15°. The mixture was permitted to stand for 1 hour, diluted with water, taken up in ether, washed thoroughly with water, dried, and evaporated to a small volume. The crystalline product after two recrystallizations from ether had a melting point of 194°. $[\alpha]_D^{20} = +83°$. $\lambda_{max.} = 243$ mμ; $\epsilon = 1.22 \times 10^4$ (absolute alcohol). Yield, 300 mg.

Analysis—$C_{24}H_{34}O_4$. Calculated, C 74.57, H 8.87; found, C 74.48, H 8.88

The methyl ester, prepared with diazomethane, melted at 130°.

*(b) From 3(α)-Hydroxy-12-keto-Δ[9,11]-cholenic Acid—*To a solution of 102 mg. of an authentic sample of 3(α)-hydroxy-12-keto-Δ[9,11]-cholenic acid in 10 cc. of acetic acid was added dropwise with stirring at 15° a solution of 50 mg. of chromium trioxide in 2 cc. of 50 per cent acetic acid. After 1 hour the mixture was worked up in the customary manner. The initial product crystallized from ether had a melting point of 193–194°, which was not depressed on admixture with a sample prepared by method (a).

*3-Keto-11,12-dibromocholanic Acid (VII)—*To a solution of 1 gm. of 3(α)-hydroxy-11,12-dibromocholanic acid in 60 cc. of acetic acid at room temperature were added 500 mg. of chromium trioxide in 10 cc. of 95 per cent acetic acid. After 1 hour the solution was diluted with water, giving 750 mg. of crystalline product, m.p. 178° with decomposition. For analysis a sample was recrystallized from ether; m.p. 178–179°.

Analysis—$C_{24}H_{33}O_2Br_3$. Calculated. C 54.14, H 6.82, Br 30.02
                              Found.   " 54.52, " 7.03, " 30.48

3-Keto-12-hydroxy-$\Delta^{9,11}$-cholenic Acid (V)—A suspension of 550 mg. of 3-keto-11,12-dibromocholanic acid in 20 cc. of water containing 1.0 cc. of 1 N sodium hydroxide and 1.6 gm. of borax was heated for 1 hour on the steam bath. Solution took place during the first 15 minutes. The mixture was then cooled and acidified with dilute hydrochloric acid; the precipitate was filtered, washed, dried, and recrystallized from ether. The pure product contained ether of crystallization, which was given up at 119–120° with partial melting, then resolidification; it finally melted at 186–187°. A mixed melting point with the ether choleic acid of II showed a depression of 15–20°. For analysis and rotation a sample was dried at 118° at 25 mm. $[\alpha]_D^{20} = +85°$. Yield, 200 mg.

Analysis—$C_{24}H_{36}O_4$. Calculated, C 74.18, H 9.34; found, C 74.22, H 9.33

Methyl 3(α),12-Diacetoxy-$\Delta^{9,11}$-cholenate (IV)—500 mg. of the ether choleic acid of 3(α),12-dihydroxy-$\Delta^{9,11}$-cholenic acid were converted to the methyl ester with diazomethane in ether-methanol. The solution was then evaporated on the steam bath. The residue was dried *in vacuo* at 100°, taken up in 0.8 cc. of pyridine and 0.7 cc. of acetic anhydride, and maintained at 80° overnight. It was then dissolved in ether, washed successively with dilute potassium carbonate, dilute hydrochloric acid, and water, dried, and evaporated on the steam bath. The residue was stirred with a few drops of dilute methanol and kept in the ice box. After a few days the crystals were filtered off and washed with cold dilute methanol. Yield, 400 mg.; m.p. 80–82°. After two recrystallizations from dilute methanol fine white needles were obtained, m.p. 84–86°. Saponification with hot methanolic sodium hydroxide and crystallization from ether gave II in quantitative yield.

Analysis—$C_{29}H_{44}O_6$. Calculated. C 71.27, H 9.09, —$COCH_3$ 17.6
                              Found.   " 71.43, " 9.05,   "   16.7

3(α),12-Dihydroxy-$\Delta^{9,11}$-cholenic Acid (II) *from III*—A solution of 300 mg. of methyl 3(α)-hydroxy-12-methoxy-$\Delta^{9,11}$-cholenate in 15 cc. of glacial acetic acid was heated on the steam bath for 16 hours. The solvent was removed *in vacuo*, and the residue taken up in 10 cc. of alcohol and 5 cc. of 20 per cent aqueous sodium hydroxide. The solution was refluxed for $\frac{1}{2}$ hour, the alcohol removed *in vacuo*, and the solution diluted with water. Dilute hydrochloric acid was added, and the amorphous precipitate filtered off, washed with water, and taken up in absolute ether. The dihydroxycholenic acid (II) separated rapidly in crystalline form as the ether choleic acid, m.p. 146–147°, followed by resolidification and remelting at 190–191°.

A mixed melting point with an authentic sample of II showed no depression. As further identification the dihydroxy acid was converted to the diacetate methyl ester which had a melting point of 84–86° and showed no depression on admixture with an authentic sample of IV.

Methyl 3(α)-Hydroxy-12-methoxy-9,11-oxidocholanate (VIII)—1.0 gm. of methyl 3(α)-hydroxy-12-methoxy-Δ⁹, ¹¹-cholenate was dissolved in 10 cc. of chloroform containing 580 mg. of perbenzoic acid. After 18 hours at 0°, 1.03 atoms of oxygen had been consumed. The chloroform solution was evaporated *in vacuo* under a small volume of water; the crystalline residue was taken up in ether, washed with dilute potassium carbonate, dried, and evaporated to a small volume. 1.0 gm. of crystalline material was obtained, m.p. 178–180°. Recrystallization from methanol raised the melting point to 180–181°. For analysis, a small sample was recrystallized several times from ether and dried at 110°. It then had a melting point of 181°.

Analysis—$C_{26}H_{42}O_5$. Calculated. C 71.86, H 9.73
Found. " 71.92, 71.93, " 9.81, 9.82

Action of Sulfuric Acid and of Acetic Anhydride on VIII—(a) 200 mg. of VIII in 13 cc. of acetone and 1 cc. of water containing 2 drops of concentrated sulfuric acid were permitted to stand at room temperature for 60 hours. At the end of this time the solution was concentrated *in vacuo*, giving a nearly quantitative return of starting material; m.p. and mixed m.p. 177–180°.

(b) 200 mg. of VIII in 13 cc. of acetone and 2 cc. of 10 per cent sulfuric acid were refluxed for 1 hour; dilution of a sample of the solution then gave starting material, identified by melting point and mixed melting point. If the refluxing was continued for 7 hours, a brownish oil was obtained which, after separation into neutral and acidic fractions, could not be obtained crystalline.

(c) 200 mg. of VIII were dissolved in 2 cc. of acetic anhydride and left on the steam bath for 2 hours. The excess acetic anhydride was then decomposed with water; the product was taken up in ether, washed with water and dilute potassium carbonate, dried, and evaporated on the steam bath. The colorless, oily acetate methyl ester was then saponified by boiling for 10 minutes with a methanolic solution of sodium hydroxide, diluted with water, and acidified with dilute hydrochloric acid; the product was taken up in ether, washed with water, dried, and evaporated. Since the acid did not crystallize at this point, it was dissolved in ether and treated with diazomethane. VIII separated immediately; m.p. and mixed m.p. 180–181°.

Methyl 3-Keto-12-methoxy-9,11-oxidocholanate (IX)—A solution of 400

mg. of VIII in 20 cc. of acetic acid was treated dropwise with stirring at 15° with 400 mg. of chromium trioxide in 14 cc. of 70 per cent acetic acid and allowed to remain at room temperature for $1\frac{1}{2}$ hours. The product, worked up in the customary manner, was crystallized from pentane and after two recrystallizations from ether-pentane had a melting point of 85°.

Analysis—$C_{26}H_{40}O_4$. Calculated, C 72.19, H 9.31; found, C 71.99, H 9.24

Oxime of IX—90 mg. of IX were refluxed with 200 mg. of hydroxylamine hydrochloride and 200 mg. of potassium acetate in 5 cc. of 60 per cent alcohol. The solution was cooled, water was added to incipient turbidity, and the mixture left in the ice box. Fine, colorless needles of oxime slowly separated; m.p. 144–145°, not raised by recrystallization.

Analysis—$C_{26}H_{41}O_5N$. Calculated. C 69.77, H 9.23, N 3.20, —OCH_3 13.8
Found.   " 69.99, " 9.50, " 3.17,   " 12.9
" 69.37, " 9.18

12-Methoxy-$\Delta^{2:\,9,11}$-choladienic Acid (XII)—10.5 gm. of III were dissolved in 45 cc. of dry pyridine with warming. The solution was then rapidly chilled to 0°, treated with a cold solution of 5.6 gm. of *p*-toluenesulfonyl chloride in 10 cc. of dry pyridine, and allowed to stand overnight at room temperature. A small volume of water was then added to decompose the excess acid chloride; the product was taken up in ether and washed successively with cold dilute hydrochloric acid, sodium carbonate, and water. After being dried over sodium sulfate, the solution was evaporated to a small volume on the steam bath, the remainder of the ether being removed *in vacuo* at room temperature. The oily tosylate was then taken up in 40 cc. of collidine and refluxed for $\frac{1}{2}$ hour. The product was aken up in ether, washed with water and dilute hydrochloric acid, againt with water, and then evaporated on the steam bath. The unsaturated methyl ester was saponified by boiling with 1.5 per cent methanolic sodium hydroxide for 1 hour. The methanol was then removed *in vacuo*, and the residual sodium salts treated with dilute hydrochloric acid and ether. The ethereal solution was washed with water and treated with 50 cc. of cold 10 per cent sodium hydroxide. The ether layer was decanted and the precipitated sodium salts filtered on asbestos and washed with 10 per cent sodium hydroxide. Acidification of the sodium salt with dilute hydrochloric acid, extraction with ether, and concentration of the solution gave pale yellow needles of the mixture of 2,3- and 3,4-unsaturated acids with a melting point varying from 150–160°. After recrystallization from dilute acetone and from ether the acid had a melting point of 162–163°; $[\alpha]_D^{20} = +99°$. Yield, 5.4 gm.

Analysis—$C_{25}H_{36}O_3$. Calculated, C 77.68, H 9.91; found, C 77.39, H 10.22

12-Methoxy-$\Delta^{9,11}$-cholenic Acid (XIII)—700 mg. of the mixture of Δ^3- and Δ^2-unsaturated acids (crystallized once from ether and having a melting point of 150–152°) were dissolved in 150 cc. of methanol and shaken with 100 mg. of platinum oxide catalyst under 30 pounds of hydrogen. After 5 minutes of shaking, slightly more than the theoretical amount of hydrogen had been taken up. The catalyst was then filtered off, the solution evaporated to a small volume *in vacuo*, and water added to turbidity. The product separated as colorless needles, m.p. 127–128°. Yield, 700 mg. After two recrystallizations from dilute alcohol the melting point was 133–133.5°. $[\alpha]_D^{20} = +141°$.

Analysis—$C_{25}H_{40}O_3$. Calculated. C 77.28, H 10.37, —OCH$_3$ 7.72
Found. " 77.24, " 10.08, " 7.84

The methyl ester, prepared with diazomethane, crystallized from dilute acetone in nearly cubical form and had a melting point of 66°.

Analysis—$C_{26}H_{42}O_3$. Calculated, C 77.57, H 10.50; found, C 77.63, H 10.70

The acid and its methyl ester are stable to hot alcoholic selenium dioxide, chromium trioxide in acetic acid at 15°, aqueous alkaline permanganate, and osmium tetraoxide in absolute ether. Hot nitric acid gave an oil.

Reaction of 12-Methoxy-$\Delta^{9,11}$-cholenic Acid with Bromine—500 mg. of acid in 1 cc. of acetic acid and 5 cc. of ether were treated with 1.1 moles of bromine in 2 cc. of acetic acid. When the theoretical amount of bromine had been consumed (about 10 minutes at room temperature), the solution was diluted with ether, washed with dilute sodium bisulfite and then exhaustively with water to remove acetic acid, dried, and concentrated *in vacuo* to a volume of about 1 cc. 3 cc. of pentane were then added and the solution permitted to stand at 0° overnight. The crystals were then filtered off and washed with pentane. Yield, 570 mg.; m.p. 122–128°. After a number of recrystallizations from ether-pentane a colorless, crystalline bromo acid with a melting point of 132–133° was obtained. Although analysis indicated that the bromine content was high, the carbon and hydrogen values were in good agreement with an empirical formula of $C_{25}H_{39}O_3Br$.

Analysis—$C_{25}H_{39}O_3Br$. Calculated. C 64.24, H 8.41, Br 17.08
Found. " 64.32, " 8.40, " 18.24

From the reaction of the bromo acid with alcoholic or aqueous sodium hydroxide no crystalline product could be obtained.

Reaction of Methyl Ester of XIII with Perbenzoic Acid—1.290 gm. of methyl 12-methoxy-$\Delta^{9, 11}$-cholenate were treated with 20 cc. of chloroform containing 66.2 mg. of perbenzoic acid per cc. After about 60 hours at

0°, the theoretical amount of oxygen had been consumed. The solution was then washed with dilute sodium carbonate and evaporated *in vacuo* under a small volume of water. The resulting gum was crystallized from cold dilute methanol. Yield, 0.5 gm.; m.p. 60–65°. After two recrystallizations from dilute acetone the colorless, cubic crystals of the oxide melted at 78–80°.

SUMMARY

1. The reactions of two derivatives of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid containing oxygen in Ring C have been investigated. On the basis of these reactions the structures of $3(\alpha)$-hydroxy-12-methoxy-$\Delta^{9,\,11}$-cholenic acid have been assigned to these derivatives.

2. 12-Methoxy-$\Delta^{9,\,11}$-cholenic acid has been synthesized and some of its reactions investigated.

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PARTIAL SYNTHESIS OF PREGNENE-4-TRIOL-17(β),20(β),21-DIONE-3,11 AND PREGNENE-4-DIOL-17(β),21-TRIONE-3,11,20 MONOACETATE

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In 1936 Kendall and coworkers (1) and Pfiffner and Wintersteiner (2) isolated from adrenal cortical extracts a compound which melted at about 215°. It was designated by the respective groups as Compound E and Substance F. Subsequently Reichstein (3) showed that Compound E and Substance F were identical with his Substance Fa and that the structure was pregnene-4-diol-17(β),21-trione-3,11,20 (II) (4).

From similar adrenal cortical extracts Reichstein and von Euw (5) isolated a compound, $C_{21}H_{30}O_5$. This compound, designated as Substance U, was shown to be pregnene-4-triol-17(β),20(β),21-dione-3,11 (I).[1]

The partial synthesis of Compound E and of Substance U from desoxycholic acid has now been accomplished in this laboratory. Methyl bisnor-desoxycholate (III) was prepared by Barbier-Wieland degradation according to the procedure of Hoehn and Mason (7). Treatment of III with benzoyl chloride gave the dibenzoate (IV) (8). The dibenzoate was partially *trans* esterified to give methyl 3(α)-hydroxy-12(β)-benzoxybisnorcholanate (V), which was pyrolyzed, giving methyl 3(α)-hydroxybisnor-$Δ^{11}$-cholenate (VI).[2] The unsaturated product was found to be most conveniently isolated as the 3-monohemisuccinate.

The general procedure of Reich and Reichstein (11) was then followed through the bromohydrin (VII) and the bromoketone (VIII) to methyl 3(α)-acetoxy-11-ketobisnorcholanate (IX) (8). Two minor changes in experimental procedure were introduced in the preparation of IX: (a) the addition of hypobromous acid to the C_{11-12} double bond was carried out in the presence of dilute sulfuric acid, thus decreasing the reaction time to

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[1] The partial syntheses of the acetates of Reichstein's Substances U and Fa have been accomplished by the oxidation of the acetates of his Substances E and M respectively (1, 6).

[2] The synthesis of this compound and its derivatives was based on a procedure of Kendall and coworkers (9) for the preparation of 3(α)-hydroxy-$Δ^{11}$-cholenic acid. The synthesis of 3(α)-hydroxybisnor-$Δ^{11}$-cholenic acid and some of its derivatives described in this paper has been accomplished by Lardon and Reichstein (8), using a somewhat similar procedure. In addition, both Kendall and coworkers (personal communication) and Grandjean and Reichstein (10) have obtained this acid by the Barbier-Wieland degradation of 3(α)-hydroxy-$Δ^{11}$-cholenic acid.

3 minutes; (b) the crude crystalline bromohydrin was given a mild treatment with zinc dust in benzene-acetic acid, converting the chief high melting contaminant, the $11,12$-dibromide, to the corresponding Δ^{11} derivative. This was then easily removed by fractional crystallization.

Saponification of IX gave $3(\alpha)$-hydroxy-11-ketobisnorcholanic acid (X). The drastic conditions required for this hydrolysis appeared to have caused slight racemization at C-20, since treatment of the total crude hydroxy acid with diazomethane gave a somewhat impure sample of methyl $3(\alpha)$-hydroxy-11-ketobisnorcholanate, previously prepared directly from IX by mild hydrolysis. Acetylation of X gave $3(\alpha)$-acetoxy-11-ketobisnorcholanic acid (XI), which was converted to $3(\alpha)$-acetoxy-11-keto-20-aminopregnane (XIV) by means of the Curtius rearrangement (12). The acid chloride (XII) reacted readily with sodium azide in dilute acetone, giving the acid azide (XIII). Decomposition (13) of the acid azide in

CH₂OH   CH₂OH

HCOH   CO

(I)   (II)

dilute acetic acid yielded XIV. Diazotization (12) of XIV in aqueous pyridine gave about 70 per cent of a mixture of pregnenes, subsequent oxidation of which indicated that the components included the Δ^{17} (XV), Δ^{20} (XVI), and Δ^{16} (XVII ?) isomers, together with about 20 per cent of C-20 alcohols (XVIII). This ratio of alcohols to unsaturated hydrocarbons is considerably greater than when dilute alcohol or acetic acid is used as a solvent. On a preparative scale, the alcohols were not separated from the mixture of unsaturated compounds but were converted via the tosylate into the same or a comparable mixture of pregnenes.

This mixture of isomeric $3(\alpha)$-acetoxy-11-ketopregnenes could be obtained crystalline, but neither by fractional crystallization nor by chromatography could its components be isolated in pure form. Consequently the mixture was subjected directly to ozonolysis, giving a mixture of aldehydes and ketones. Mild permanganate oxidation of this product converted the aldehydes to carboxylic acids. From this acid fraction direct

(III) → ϕCOCl → (IV) → KOH →

(V) → Δ then Ac$_2$O → (VI) → HOBr →

(VII) → CrO$_3$ → (VIII) → Zn →

(IX) $\xrightarrow{\text{KOH}}$ (X) $\xrightarrow{\text{HOAc}}$

(XI) $\xrightarrow{\text{SOCl}_2}$ (XII) $\xrightarrow{\text{NaN}_3}$

(XIII) $\xrightarrow[\text{HOAc}]{\text{Dilute}}$ (XIV)

XXI $\xrightarrow{\text{KOH}}$ (XXII) $\xrightarrow{\text{C}_2\text{H}_2}$ (XXIII) $\xrightarrow{\text{H}_2}$

(XXIV) $\xrightarrow{\text{Ac}_2\text{O}}$ (XXV) $\xrightarrow{\text{PBr}_3}$

(XXVI) $\xrightarrow{\text{KOAc}}$ (XXVII) $\xrightarrow{\text{KOH}}$

(XXVIII) $\xrightarrow[\text{anhydride}]{\text{Succinic}}$ (XXIX) $\xrightarrow[\text{then KOH}]{\text{CrO}_3}$

(XXX) → (XXXI) → (XXXII) → (XXXIII) → (I) → (XXXIV)
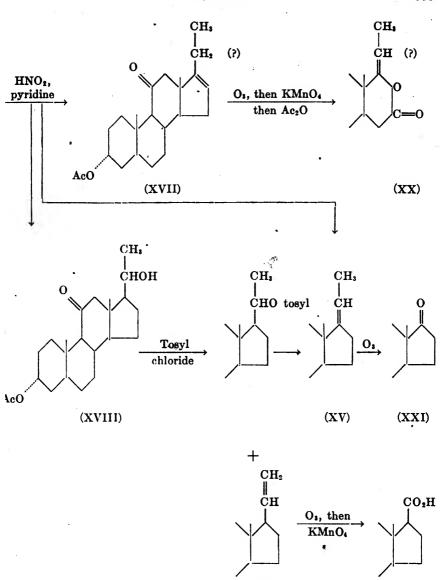
(XXXV)   (XXXVI)

crystallization gave 3(α)-acetoxy-11-ketoetiocholanic acid (XIX). Acetylation[3] of the mother liquors gave a neutral compound, $C_{23}H_{32}O_5$, to which the tentative structure XX has been assigned.

The ketones remaining in the neutral fraction were separated by means of Girard's Reagent T (14). The product so obtained could be crystallized directly to give a compound, $C_{21}H_{30}O_4$, to which has been assigned the structure etiocholanol-3(α)-dione-11,17 acetate (XXI). Chromatography of the mother liquors gave a small amount of ketonic product of undetermined structure.

Alkaline saponification of XXI gave the corresponding hydroxy diketone (XXII). It was found that application of the Nef reaction (cf. (15)) to this compound resulted in addition of acetylene exclusively at C-17. A good yield of the compound $C_{21}H_{30}O_3$ (XXIII) was obtained. Partial reduction (cf. (16)) of this pregninediolone gave the corresponding ethylene, pregnene-20-diol-3(α),17(α)-one-11 (XXIV). The 3-monoacetate (XXV) was then converted through the crystalline intermediates 21-bromopregnene-17-ol-3(α)-one-11 acetate (XXVI) and pregnene-17-diol-3(α),21-one-11 diacetate (XXVII) to pregnene-17-diol-3(α),21-one-11 (XXVIII). The method used followed the general procedure of Ruzicka and Müller (17) for the conversion of pregnadiene-4,20-ol-17(α)-one-3 to pregnadiene-4,17-ol-21-one-3.

The conversion of the C-3-hydroxyl group to the ketone, which had to be carried out at some phase of the synthesis, was best accomplished with the diol, XXVIII. Although a number of possible methods had been investigated at the stages represented by XXIV and XXVI, the results were uniformly unsatisfactory. The partial succinoylation of XXVIII, however, proceeded very smoothly to give the C-21-monohemisuccinate (XXIX), which could readily be oxidized with chromic acid and saponified to the desired pregnene-17-ol-21-dione-3,11 (XXX).

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[3] For the technique of acetylation used, we are indebted to Dr. Whitman and Dr. Schwenk of the Schering Corporation (personal communication).

Hydroxylation of the acetate of pregnene-17-ol-21-dione-3,11 by the method of Criegee (18) gave exclusively a trioldione isolated as the diacetate (XXXI), which, by analogy with the results of Serini, Logemann, and Hildebrand (19), could immediately be assigned the structure pregnanetriol-17(β),20(β),21-dione-3,11.[4]

The conversion of XXXI into the corresponding Δ^4-unsaturated compound was accomplished by bromination at C-4 followed by removal of hydrogen bromide (cf. (21)). The pregnene-4-triol-17(β),20(β),21-dione-3,11 diacetate-20,21 (XXXIII) and the free pregnenetrioldione (I) ob-

CH$_2$OH   CH$_2$OAc

HCOH   HCOH

1Ac$_2$O   CrO$_3$

(XXXVII)   (XXXVIII)

CH$_2$OAc
CO

+

(XXXIX)   (XL)

tained by this method appeared to be identical with Reichstein's Substance U diacetate and free U respectively. Although no direct comparison was possible, the agreement of physical constants[5] was such that reasonable doubt was eliminated.

It has been reported in many examples in the literature that the chromic acid oxidation of 17,20-dihydroxypregnanes leads to 17-ketones with complete loss of the side chain (cf. (22)). However, it was found that under

[4] The configurational designations used here follow the convention of Reichstein et al. (20).

[5] See the experimental part (cf. (5)).

suitable conditions this oxidizing agent gave a very appreciable yield of the 17-hydroxy-20-ketopregnane. Investigation of the mild chromic acid oxidation of pregnene-4-triol-17(β),20(β),21-one-3 ((23), *cf.* (24)) monoacetate-21 (XXXVIII) showed that pregnene-4-diol-17(β),21-dione-3,20 monoacetate-21 (Reichstein's Substance S monoacetate, the isolation and partial synthesis (25) of which have been described by Reichstein and von Euw) (XXXIX) was formed. In addition the expected product, androstene-4-dione-3,17 (XL), was obtained.

By this procedure the free Substance U was converted to the 21-monoacetate (XXXIV). Oxidation then gave a mixture of adrenosterone (26) (XXXV) and pregnene-4-diol-17(β),21-trione-3,11,20 monoacetate-21 (XXXVI). The identity of the latter compound was established by comparison with an authentic sample of Compound E monoacetate, which was kindly furnished us by Dr. Kendall.

The author wishes to express his appreciation to Dr. Everett S. Wallis of Princeton University and to Dr. Karl Folkers and Dr. Randolph T. Major for their active association with this work. The author is indebted to Dr. Jacob van de Kamp for the preparation of methyl 3(α)-acetoxybisnor-Δ^{11}-cholenate and for various improvements in this preparative procedure. The author wishes to express his appreciation to Dr. Ralph Mozingo and to Mr. William Wright for their work in connection with the catalytic reduction described in this paper.

<center>EXPERIMENTAL[6, 7]</center>

Methyl 3(α),12(β)-Dibenzoxybisnorcholanate (IV)—A solution of 50 gm. of bisnordesoxycholic acid hydrate in 1500 cc. of methanol and 15 cc. of concentrated sulfuric acid was refluxed overnight. The solution was then concentrated to half volume, diluted with water, and taken up in ether. The washed ethereal solution was treated with a small volume of ethereal diazomethane to complete the esterification. The ethereal solution was then concentrated to dryness, finally *in vacuo* on the steam bath. Traces of methanol were removed by dissolving the residue in 50 cc. of pyridine and concentrating to dryness *in vacuo*. The crude methyl ester was then dissolved in 150 cc. of pyridine and treated with 41 cc. of benzoyl chloride. The mixture was heated on the steam bath for 2 hours, treated with 5 cc. of water, cooled, diluted, and extracted with ether. The ethereal solution was washed with dilute hydrochloric acid, dilute sodium hydroxide, water,

and then taken to dryness on the steam bath. The dibenzoate was recrystallized from alcohol; m.p. 173.5°; yield 90 per cent.

Methyl 3(α)-Hydroxy-12(β)-benzoxybisnorcholanate (V)—A solution of 100 gm. of dibenzoate methyl ester in 500 cc. of benzene at 24–25° was treated with 500 cc. of a solution of 1.11 N potassium hydroxide in dry methanol. The solution was swirled in a water bath at 24–25° until it was homogeneous and then left to stand in the water bath at 24–25° for 90 minutes. A few drops of phenolphthalein were added and then sufficient glacial acetic acid to neutralize the base. The solvent was removed *in vacuo*, the residue dissolved in ether, and the ethereal solution washed with dilute sodium carbonate and then several times with water. (No perceptible amount of benzoic acid or bisnor acids was found in the alkaline washings.) The ethereal solution was concentrated to dryness, finally *in vacuo*, on the steam bath, leaving a mixture of methyl benzoate and the crude hydroxy benzoxy methyl ester as a pale yellow oil.

Methyl 3(α)-Acetoxybisnor-Δ¹¹-cholenate (VI)—The crude hydroxy benzoxy methyl ester obtained from 100 gm. of dibenzoate was transferred to a Claisen flask, a few boiling chips were added, and the flask then heated in a metal bath under a vacuum of 15 to 20 mm. From about 190–210° methyl benzoate distilled and was collected separately. The bath temperature was then raised until the temperature of the distillate vapor was 315°. A mixture of benzoic acid and unsaturated methyl ester was then steadily distilled off for 25 minutes. The product was then cooled to about 125° and poured into benzene. The flask, including the benzoic acid distillate, was then washed out with benzene. The combined benzene solutions were washed with dilute alkali and with water and evaporated *in vacuo*.

The product so obtained consisted roughly of 45 per cent of starting material, 40 per cent of the desired unsaturated material, and the remainder of by-products. The starting material was first removed by conversion to the acetoxy benzoxy methyl ester; the residue from the benzene extraction was taken up in a mixture of 150 cc. of pyridine and 100 cc. of acetic anhydride and heated on the steam bath for 45 minutes. The product was then treated with water, extracted with ether, the ethereal solution washed with dilute hydrochloric acid, dilute alkali, and finally with water, and evaporated to dryness. The residue was dissolved in a small volume of methanol with refluxing and the methyl 3(α)-acetoxy-12(β)-benzoxybisnorcholanate permitted to crystallize overnight at room temperature. The crystalline product was then filtered; m.p. 166–168°; yield 30 to 35 gm. This material was then subjected to partial *trans* esterification as before, except that the time required was 10 minutes at 24–25° rather than 90 minutes.

The mother liquors obtained from the acetoxy benzoxy methyl ester were concentrated to dryness *in vacuo* and then dissolved in 300 cc. of benzene. To this solution were added 300 cc. of 1.1 N methanolic potassium hydroxide and the whole permitted to stand at 25° for 10 minutes. The mixture was then acidified with glacial acetic acid and worked up as described above. The crude oily methyl 3(α)-hydroxybisnor-Δ^{11}-cholenate so obtained was dissolved in 250 cc. of pyridine and treated with 60 gm. of succinic anhydride. The solution was heated on the steam bath for 90 minutes, the excess succinic anhydride decomposed with water, and the solution concentrated to a small volume *in vacuo*. The residue was diluted, extracted with ether, and the ether layer washed with dilute hydrochloric acid and water. The monohemisuccinate was then separated from small amounts of non-acidic impurities by extraction with a 10 per cent solution of potassium carbonate, acidification of the alkaline layer, and again dissolving in ether. ·The ethereal solution was then washed and concentrated to dryness on the steam bath. The partially crystalline residue was then recrystallized once from methanol or benzene and a second time from benzene or acetone. The methyl 3(α)-succinoxybisnor-Δ^{11}-cholenate had a melting point of 187–189°; yield 30 per cent.

By concentration of the mother liquors a small additional quantity of succinate was obtained. Mild acid or alkaline hydrolysis of the mother liquors followed by rebenzoylation gave crystalline dibenzoate which was again partially *trans* esterified and pyrolyzed. The total yield thus obtained was 60 per cent.

The succinoxy group was then removed either by alkaline (*a*) or acid (*b*) hydrolysis. (*a*) A solution of 27 gm. of the Δ^{11}-succinoxy ester in 1300 cc. of methanol containing 27 gm. of sodium hydroxide was refluxed for 75 minutes, concentrated *in vacuo* to 500 cc., diluted with a large volume of water, and the crystalline product filtered and dried. (*b*) The Δ^{11}-succinoxy ester was refluxed in a 2 per cent (by weight) methanolic solution of sulfuric acid for 2 hours, water was added to the cooled solution, and the crystals filtered and dried. The methyl 3(α)-hydroxybisnor-Δ^{11}-cholenate prepared by either method melted at 108°. Acetylation with equal parts of pyridine and acetic anhydride at 90° for 30 minutes, followed by cooling and dilution with water, gave the crystalline acetate. Recrystallization from a small volume of methanol gave the pure methyl 3(α)-acetoxybisnor-Δ^{11}-cholenate, m.p. 101–102°, in a yield of 85 to 90 per cent (based on the succinoxy derivative). The product gave no melting point depression with a sample prepared by Barbier-Wieland degradation of 3(α)-hydroxy-Δ^{11}-cholenic acid.

Methyl 3(α),11-Dihydroxy-12-bromobisnorcholanate (*VII*)—A solution of 15.8 gm. of methyl-3(α)-acetoxybisnor-Δ^{11}-cholenate in a mixture of

430 cc. of tertiary. butyl alcohol and 105 cc. of water was cooled to 15°. 12 gm. of anhydrous N-bromoacetamide were then dissolved with swirling and 105 cc. of cold 0.8 N sulfuric acid added at once. The mixture was permitted to stand at room temperature until a yellow color developed (2 to 4 minutes). An aqueous solution of sodium sulfite was then added, sufficient to discharge the yellow-orange coloration, a large volume of water was added, and the mixture was extracted twice with benzene. The benzene layer was washed with dilute sodium hydroxide, several times with water, and concentrated to a small volume *in vacuo*. The addition of petroleum ether (30–60°) precipitated crude crystalline bromohydrin (12.0 to 12.5 gm.). This material was purified by dissolving it in a mixture of 135 cc. of benzene and 15 cc. of glacial acetic acid, adding 10 gm. of zinc dust, and swirling for a minute at room temperature. The solution was then diluted with ether, decanted from the zinc, washed with water, dilute alkali, again with water, then concentrated *in vacuo* to a small volume, and crystallized with the addition of petroleum ether. One additional crystallization gave a product of melting point 209–211°; yield 45 per cent.

Analysis—$C_{25}H_{39}O_5Br$.   Calculated.   C 60.12, H 7.87, Br 16.00
　　　　　　　　　　　　　Found.　·　" 59.80, " 7.71, " 16.48

Methyl 3(α)-Acetoxy-11-keto-12-bromobisnorcholanate (VIII)—10 gm. of methyl 3(α)-acetoxy-11-hydroxy-12-bromobisnorcholanate were dissolved in 150 cc. of glacial acetic acid by warming on the steam bath. The solution was then cooled rapidly to 18° and treated portionwise, with stirring, with a solution of 5 gm. of chromic acid in 100 cc. of acetic acid and 10 cc. of water. The mixture was permitted to stand at room temperature for 90 minutes and the product then precipitated by the addition of water. It had a melting point of 154.5–155.0°; yield 92 per cent.

Methyl 3(α)-Acetoxy-11-ketobisnorcholanate (IX)—A solution of 7.6 gm. of methyl 3(α)-acetoxy-11-keto-12-bromobisnorcholanate in 70 cc. of glacial acetic acid and 7 cc. of water was warmed on the steam bath to 60°. The mixture was then treated portionwise with 7.0 gm. of zinc dust. The temperature was allowed to rise to 90–95° and the mixture was stirred for 1 hour. It was then cooled, diluted, and extracted with benzene. The benzene layer was washed with water, dilute alkali, again with water, and concentrated to dryness *in vacuo*. The residue was crystallized from methanol and melted at 151.0–151.5°; $[\alpha]_D = +57°$. A sample crystallized from dilute acetic acid melted at 117.5–118.0°. Another sample which was crystallized from dilute methanol melted at 142.5–143.5°, resolidified, and melted at 150.5–151.5°.

Analysis—$C_{25}H_{38}O_5$.   Calculated C 71.74, H 9.15; found, C 71.53, H 9.33

Methyl 3(α)-Hydroxy-11-ketobisnorcholanate—A sample of methyl 3(α)-acetoxy-11-ketobisnorcholanate (IX) was refluxed with a 1.5 per cent solution of sodium hydroxide in methanol for 1 hour. Dilution with water gave a crystalline product which, after one recrystallization from acetone-pentane, melted at 193°. Reacetylation of a small sample gave a product which was crystallized from dilute methanol and melted at 142.5–143.5° and 150.5–151.5°; it did not depress the melting point of a sample of IX.

Analysis—$C_{23}H_{36}O_4$. Calculated, C 73.36, H 9.64; found, C 73.37, H 9.84

3(α)-Hydroxy-11-ketobisnorcholanic Acid (X)—A solution of 1.0 gm. of methyl 3(α)-acetoxy-11-ketobisnorcholanate (IX) in 25 cc. of a 2 N potassium hydroxide-75 per cent methanol mixture was refluxed overnight. Most of the methanol was removed *in vacuo*, and the solution diluted with water and acidified with dilute hydrochloric acid. The amorphous precipitate was filtered, washed, and dried. It melted at about 195° and 223–225°. On a preparative scale no further purification was attempted, since recrystallization did not seem to improve the quality of the product.

Methyl 3(α)-Hydroxy-11-ketobisnorcholanate from X—A sample of crude 3(α)-hydroxy-11-ketobisnorcholanic acid (m.p., about 222–225°) was suspended in absolute ether and treated with an ethereal solution of diazomethane. The solution was concentrated to dryness after the acid had dissolved and the methyl ester crystallized by rubbing with pentane. The crystals were washed with a little pentane; they melted at 187–190°. They did not depress the melting point of methyl 3(α)-hydroxy-11-ketobisnorcholanate.

3(α)-Acetoxy-11-ketobisnorcholanic Acid (XI)—A solution of 3 gm. of 3(α)-hydroxy-11-ketobisnorcholanic acid in 100 cc. of glacial acetic acid was refluxed overnight. The solution was then concentrated *in vacuo* to 30 cc., warmed on the steam bath, and treated with small portions of water until crystallization began. It was set aside at 0° overnight and the product filtered off. It had a melting point of 239–250° and was sufficiently pure for subsequent degradation to the mixture of 3-acetoxy-11-ketopregnenes; yield 90 per cent. Several recrystallizations from dilute acetone gave material still characterized by the wide melting range, 249–254°.

3(α)-Acetoxy-11-keto-20-aminopregnane (XIV)—30 gm. of 3(α)-acetoxy-11-ketobisnorcholanic acid were dissolved in 45 cc. of purified thionyl chloride. The solution was left to stand at room temperature for 90 minutes, was warmed to 45° for 30 minutes, and then concentrated to dryness *in vacuo*. The residue was dissolved in 45 cc. of warm absolute toluene and the latter distilled off *in vacuo*, finally at 100°. The residual crystalline acid chloride (XII) (m.p. 140–144°) was then dissolved in 200 cc. of dry acetone at 5–10°, cooled to 0°, and treated with a cold solution of 12 gm.

of sodium azide in 30 cc. of water, added at once. The mixture was swirled gently for 5 minutes in an ice bath, at the end of which time crystallization of the acid azide (XIII) had begun. The precipitation was completed by the gradual addition of 700 cc. of ice water, and the crystals filtered on a cold filter, washed with ice water, and then transferred to a flask containing 360 cc. of glacial acetic acid. The solution was warmed on the steam bath to dissolve the azide while 200 cc. of water were gradually added. Finally the decomposition of the azide was completed by heating for 30 minutes at 90°. The solution was then cooled in an ice-salt bath, 100 cc. of benzene were added, and the mixture treated gradually, with stirring, with a cold solution of 425 gm. of potassium hydroxide in 700 cc. of water. The temperature was maintained at 10° or lower in order to minimize saponification of the C-3-acetoxy group. When the alkali had all been added, the benzene layer was diluted with ether and the aqueous phase was extracted. At the ether-alkali interface a solid precipitate separated which was collected separately. It consisted of the potassium salt of XI (about 1.5 gm.) and 3(α)-hydroxy-11-keto-20-aminopregnane (about 200 mg.). This mixture was readily separated by dissolving the wet alkaline precipitate in a little water, filtering the solution, acidifying the filtrate to obtain crude XI, and collecting the water-insoluble residue on the filter to obtain the hydroxy keto amine. The washed ether-benzene layer containing XIV was concentrated, finally *in vacuo* on the steam bath, and the residue dissolved in 100 cc. of absolute ether. The solution was allowed to crystallize at 0° overnight, filtered, and a second crop obtained by concentration of the mother liquors; yield 22.6 gm. = 80 per cent (corrected for recovered starting material). The mother liquors were treated with hydrogen chloride in absolute ether. The amorphous hydrochlorides so obtained (combined yield 93 per cent) were diazotized (see below) and the ozonized product gave nearly as good a yield of the 17-ketone (XXI) and etio acid (XIX) as did the pure crystalline amine. Consequently the non-crystalline amines probably consisted largely of isomers of XIV resulting from the partial racemization of the side chain during the saponification of IX.

A sample of XIV recrystallized from absolute ether showed a constant melting point of 163.5–165.5°.

Analysis—$C_{23}H_{37}O_3N$. Calculated. C 73.56, H 9.94, N 3.73
Found. " 73.88, " 10.06, " 3.73

3(α)-Hydroxy-11-keto-20-aminopregnane, 3(α)-Acetoxy-11-keto-20-acetylaminopregnane, and 3(α)-Hydroxy-11-keto-20-acetylaminopregnane—For purposes of further characterization of XIV, the hydroxy keto amine was prepared by alkaline saponification of XIV. It had a melting point of 185–186° after crystallization from dilute alcohol. Acetylation of the

hydroxy or acetoxy keto amine with pyridine-acetic anhydride gave the acetoxy keto acetylamino derivative, m.p. 235°, alkaline saponification of which gave 3(α)-hydroxy-11-keto-20-acetylaminopregnane, m.p. 219.5°.

Pregnene-16-ol-3(α)-one-11 Acetate (XVII), *Pregnene-17-ol-3(α)-one-11 Acetate (XV)*, *and Pregnene-20-ol-3(α)-one-11 Acetate (XVI)*—A mixture of 57.8 gm. of 3(α)-acetoxy-11-keto-20-aminopregnane (XIV), 250 cc. of pyridine, 21.3 gm. of redistilled pyridine hydrochloride, and 44 gm. of sodium nitrite was heated to 75° on the steam bath. To this solution were then added 150 cc. of water in one portion. The mixture was then heated on the steam bath with occasional swirling until the evolution of nitrogen ceased (14 minutes). The pyridine was then largely removed *in vacuo*, and the residue diluted with water and taken up in ether. The ethereal solution was washed with water, 0.5 N hydrochloric acid, again with water, and concentrated to dryness, finally *in vacuo* on the steam bath.

In order to remove the undiazotized 20-amino compound and 3(α)-hydroxy compounds resulting from hydrolysis during diazotization, the mixture was subjected to a mild succinoylation (C-20(α)- and (β)-hydroxy compounds remain essentially unattacked). The total crude diazotization product described above was dissolved in 250 cc. of dry pyridine, treated with 50 gm. of succinic anhydride, and heated on the steam bath for 1 hour. Water was then added to decompose the excess anhydride, and the mixture concentrated *in vacuo* to a small volume, diluted, dissolved in ether, and washed with dilute hydrochloric acid and with water.

The acid succinates were then separated by shaking with a 10 per cent solution of potassium carbonate. Saponification of the acid succinates by the addition of a small amount of concentrated potassium hydroxide to the carbonate extract and heating on the steam bath gave 5.5 gm. of material, separated by extraction with ether into 3.8 gm. of 3(α)-hydroxy-11-keto-20-aminopregnane and 2.7 gm. of oily ether-soluble hydroxyketopregnene mixture.

The neutral ethereal solution after the carbonate washing was shaken with water and concentrated to dryness, finally *in vacuo*, on the steam bath. The reddish yellow oil so obtained (51 gm.) consisted chiefly of the pregnene mixture (XV, XVI, and XVII) together with some 20-hydroxy compound (XVIII). The composition of the mixture may be roughly determined by chromatography[8] of a small sample, whereby the acetoxyketopregnenes are eluted by a 1:1 mixture of ether-petroleum ether (30–60°)

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[8] The alumina used in the various chromatograms described was especially prepared. An aqueous suspension of alumina (Merck, according to Brockmann) was treated with 6 N sulfuric acid, with stirring, until the pH was 4.0. The alumina was then washed repeatedly with distilled water until the pH of the supernatant liquid was 4.6 to 4.8. The alumina was filtered and dried overnight at 150°.

and the C-20 hydroxy compounds are completely eluted only with absolute ether.

The C-20 hydroxy compounds present in the purified diazotization product described above were then converted to their tosyl esters. The purified diazotization product (51 gm.) was dissolved in 60 cc. of dry pyridine and treated with 34.5 gm. of recrystallized *p*-toluenesulfonyl chloride. The mixture was left to stand at room temperature for 48 hours, then treated with a small volume of water to decompose the excess acid chloride, taken up in ether, washed with water and dilute sulfuric acid, again with water, and concentrated to dryness. The residual mixture of pregnenes and tosylates, 54.2 gm. of a brown oil, was dissolved in 140 cc. of collidine and the mixture was refluxed for 25 minutes. After this had been taken up in ether, washed with dilute hydrochloric acid, then with water, and finally evaporated to dryness, 48.9 gm. of a brown, glassy residue were obtained. This material was dissolved in a small volume of methanol from which, by careful cooling, 24.0 gm. of a crystalline mixture of acetoxyketopregnenes were obtained; m.p. about 75–85°. The 24.8 gm. of mother liquors were chromatographed over 250 gm. of alumina. All material eluted by 8:2 petroleum ether-ether (21.5 gm.) was combined with the 24.0 gm. of crude crystalline pregnenes; yield 88 per cent.

3(α)-Acetoxy-11-ketoetiocholanic Acid (XIX), Etiocholanol-3(α)-dione-11,17 Acetate (XXI), and Other Ozonolysis Products—The ozonolysis of the purified mixture of pregnene-16-, pregnene-17-, and pregnene-20-ol-3(α)-one-11 acetates (XVII, XV, and XVI) was conducted in 5.0 gm. portions. This amount was dissolved in a mixture of 100 cc. of ethyl acetate and 100 cc. of methanol, cooled in a bath of dry ice and acetone, and treated over a period of 2 to 3 hours with 1.5 to 2.0 moles of ozone. An apparatus with a sintered glass gas diffusion inlet was used. The solution was then treated with 40 cc. of dilute acetic acid and 5 gm. of zinc dust, warmed to room temperature, decanted from unchanged zinc, and concentrated *in vacuo* to a small volume. The residue was taken up in ether and washed with water and dilute potassium carbonate. The acids found in the aqueous alkaline layer were present only in traces and were discarded. The washed neutral ether layer was evaporated to dryness, dissolved in 125 cc. of acetone, cooled to 15°, and treated with 35 cc. of a 5 per cent aqueous potassium permanganate solution. The mixture was permitted to stand at room temperature for 1 hour, then concentrated by directing a stream of filtered air upon the surface of the cool (15–20°) solution. When the acetone had almost entirely evaporated, the mixture was diluted with water, taken up in ether, and the manganese salts reduced with a dilute solution of sulfurous acid. The ether layer was washed, then extracted with 10 per cent potassium carbonate, which was immediately separated, acidified, and extracted with ether.

Separation of Acids—The washed ether layer containing the organic acids from the permanganate oxidation was concentrated to dryness on the steam bath, giving 2.2 to 2.6 gm. of crude acidic material which was then crystallized from dilute acetone in the refrigerator overnight. The crystals were filtered off and recrystallized from acetone-pentane. The melting point of the 3(α)-acetoxy-11-ketoetiocholanic acid is 219–221°.

Analysis—$C_{22}H_{32}O_5$.   Calculated. C 70.17,   H 8.57
                                    Found.   " 69.93, 70.08, " 8.42, 8.31

The mother liquors from the first crystallization of the acid fraction resulting from the ozonolysis of 44 gm. of pregnene mixture were combined and weighed 9.2 gm. This material was dissolved in 100 cc. of methanol containing 1.0 cc. of concentrated sulfuric acid and refluxed for 30 minutes. The mixture was then concentrated *in vacuo* and separated into neutral (Fraction N = 6.0 gm.) and acidic (Fraction A = 3.4 gm.) fractions.

The neutral Fraction N contained most of the etio acid. The methyl esters of Fraction N were separated into "ketonic" (2.6 gm.) and "non-ketonic" fractions (3.4 gm.) by the use of Girard's Reagent T, applied in the manner described below. Saponification of the neutral "non-ketonic" material followed by trituration of the resulting hydroxy acids with ethyl acetate gave 1.40 gm. of a microcrystalline highly insoluble product, 3(α)-hydroxy-11-ketoetiocholanic acid. This was not further purified but converted directly into XIX by refluxing with glacial acetic acid overnight. The purified acetoxy etio acid so obtained melted at 219–221° after crystallization from a small volume of ethyl acetate. The total yield of pure acetoxy etio acid was 24 per cent.

The 3.4 gm. of acidic Fraction A were dissolved in 15 cc. of glacial acetic acid containing 1.5 cc. of 70 per cent perchloric acid. To this were slowly added, with cooling and stirring, 12 cc. of acetic anhydride. The solution was permitted to stand at room temperature for 15 minutes, the excess acetic anhydride was carefully decomposed with water, and the product separated into neutral and acidic fractions. The neutral fraction gave, after several recrystallizations from ether, a product melting at 230–231°. The analysis indicated the empirical formula $C_{21}H_{29}O_4 \cdot CH_3CO$ (XX ?).

Analysis—$C_{23}H_{32}O_5$.   Calculated. C 71.09, H 8.28, CH_3CO 11.1
                                    Found.   " 70.94, " 8.08,   "   9.9
                                               " 70.99, " 7.87

Alkaline saponification gave an amorphous acid which on reacetylation with acetic anhydride and perchloric acid restored the neutral product melting at 223–224° in good yield.

Separation of Ketones—The ether layer containing the neutral products of ozonolysis and subsequent permanganate oxidation was washed and con-

centrated to dryness *in vacuo*. Three such neutral fractions obtained from the ozonolysis of three 5 gm. portions of pregnene mixture were combined; total weight, 7.8 gm. The ketones were then separated by taking the neutral product up in 50 cc. of methanol and 5 cc. of glacial acetic acid, adding 10 gm. of Girard's Reagent T, and refluxing for 30 minutes. The solution was then cooled in an ice bath and poured into a separatory funnel containing ether, 36 cc. of 2.0 N sodium hydroxide, water, and powdered ice. The cold aqueous layer was extracted twice with ether, then acidified to Congo red with 5 N hydrochloric acid. After standing at room temperature overnight, the partially crystalline ketonic product was separated by extraction with ether. The washed ethereal solution was evaporated to dryness and the partially hydrolyzed acetoxy group replaced by heating with pyridine and acetic anhydride on the steam bath for 15 minutes. The product was taken up in ether, washed successively with dilute hydrochloric acid, dilute alkali, and water, and evaporated to dryness, giving 4.4 gm. of crude acetylated ketonic material. This was recrystallized several times from ethyl acetate-pentane, giving long needles of etiocholanol-3(α)-dione-11,17 acetate (XXI). It had a melting point of 162.5–163.0°; $[\alpha]_D^{20} = +176°$. Chromatography of the mother liquors raised the total yield of pure product to 22 per cent.

Analysis—$C_{21}H_{30}O_4$. Calculated. C 72.80,   H 8.73
                              Found.   " 72.96, 72.50, " 8.44, 8.64

From the chromatographic separation of the mother liquors of XXI, a compound slightly less easily eluted was obtained in small amount; m.p. 209.0–209.5°. It showed no ultraviolet absorption maximum above 225 mμ.

Analysis—Found, C 71.17, 71.15, H 8.72, 8.92, —OMe 0.0

Mild alkaline saponification converted it to an amorphous neutral product which on reacetylation gave a difficultly crystalline mixture of acetates which was not further investigated.

Etiocholanol-3(α)-dione-11,17 .(*XXII*)—A solution of 380 mg. of etiocholanol-3(α)-dione-11,17 acetate (XXI) in 4 cc. of 2 N 75 per cent dilute methanolic potassium hydroxide was refluxed for 15 minutes. The addition of water gave a quantitative yield of crystals, m.p. 185–186.5°. A sample recrystallized for analysis from acetone-pentane melted at 187–188°.

Analysis—$C_{19}H_{28}O_3$. Calculated, C 74.97, H 9.26; found, C 74.93, H 9.26

Pregnine-20-diol-3(α),17(α)-one-11 (*XXIII*)—Pure dry acetylene was passed into a solution of 1.3 gm. of potassium metal in 30 to 35 cc. of anhydrous liquid ammonia maintained in a dry ice-acetone bath. The stream of

acetylene was continued after the complete decolorization of the solution for a length of time equal to that required for the decolorization. To the suspension of potassium acetylide was then added a solution of 500 mg. of etiocholanol-3(α)-dione-11,17 (XXII) in 10 cc. of pure dry dioxane and 10 cc. of absolute ether. The ammonia was then permitted to evaporate at room temperature, an additional 17 cc. of absolute ether was added, and the flask stoppered from the air and permitted to stand overnight at room temperature. The mixture was then poured into water, and the suspension acidified with dilute sulfuric acid and extracted twice with ether. The ether layer was washed twice with 10 per cent potassium carbonate solution, then with water, and concentrated to dryness. Crystallization from ethyl acetate gave a product melting at 218.5–219.0°; $[\alpha]_D = -9.4°$. The mother liquors were chromatographed and the combined yield of pure material was 80 per cent. No crystalline product other than XXIII was isolated.

Analysis—$C_{21}H_{30}O_2$. Calculated, C 76.33, H 9.15; found, C 76.41, H 9.07

Pregnene-20-diol-3(α),17(α)-one-11 (XXIV)—A suspension of 300 mg. of a 2 per cent palladium-barium carbonate catalyst in 50 cc. of absolute alcohol was shaken under 1 atmosphere of hydrogen until equilibrium was reached. To this suspension were then added 658 mg. of pregnine-20-diol-3(α),17(α)-one-11 and the solution shaken under hydrogen until 1 mole was absorbed. The initial rate of absorption was 11 cc. per minute. The final rate was 1.5 cc. per minute. The solution was filtered, concentrated *in vacuo* to a small volume, and crystallized by addition of water. The fluffy crystals so obtained were hydrated and melted at about 110° with loss of water. Recrystallization from dilute methanol gave crystals which, if heated rapidly, melted at 113–115°, resolidified, and melted again at 182–184°. Further recrystallization did not appear to effect appreciable purification. The water of crystallization apparently was lost by heating at 100° *in vacuo*, since a sample so treated did not show the lower melting point but only the upper. A sample dried at 110° had $[\alpha]_D = +43°$.

Analysis—$C_{21}H_{32}O_3$. Calculated. C 75.85,   H 9.70
                              Found.   " 76.05, 76.32, " 9.79, 9.87

Pregnene-20-diol-3(α),17(α)-one-11 Monoacetate-3 (XXV)—On a preparative scale the purification of the ethylene compound was undertaken after acetylation to the 3-monoacetate. The crude pregnene-20-diol-3(α),17(α)-one-11 obtained by the reduction of 3.81 gm. of the corresponding acetylene (XXIII) in 150 cc. of absolute alcohol with 200 mg. of 2 per cent palladium-barium carbonate catalyst was crystallized as described above and then dried at 100°. The 3.85 gm. of product so obtained were

combined with 0.55 gm. of similar material obtained from a previous reduction, and the whole dissolved in a mixture of 20 cc. of acetic anhydride and 15 cc. of pyridine and left at room temperature overnight. The solution was diluted with water, extracted with ether, and washed successively with dilute hydrochloric acid, dilute alkali, and water. Evaporation of the ethereal-solution gave 4.85 gm. of a crystalline residue consisting of crude XXV. After several recrystallizations from ethyl acetate-pentane, a product was obtained which melted at 187.5–189.0°. Chromatography of the mother liquors gave an additional yield, bringing the total of material of this purity to 87 per cent. This melting point did not represent the highest attainable, however; several highly purified fractions from the chromatogram melted at 189.5–191.0°.

Analysis—$C_{23}H_{34}O_4$. Calculated, C 73.75, H 9.16; found, C 73.81, H 8.87

21-Bromopregnene-17-ol-3(α)-one-11 Acetate (XXVI)—To a solution of 120 mg. of freshly redistilled phosphorus tribromide in 0.74 cc. of dry chloroform,[9] maintained at −60° in a dry ice bath, was added dropwise a solution of 400 mg. of pregnene-20-diol-3(α),17(α)-one-11 monoacetate-3 (XXV) in 5.8 cc. of dry chloroform containing 2 drops of pyridine. The solution was then allowed to stand at room temperature overnight. A small volume of water was added, the chloroform removed *in vacuo* at 15–25°, and the gummy residue dissolved in ether. The ethereal solution was washed with water, then with dilute sodium bicarbonate, again with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo* to give a colorless oil. This was dissolved in a small volume of petroleum ether (30–60°) and allowed to crystallize at 0°. By this means 250 mg. of crude crystalline product were obtained. A small sample was recrystallized with difficulty from ether-methanol by concentration *in vacuo* and recrystallized again from ether-pentane by concentration *in vacuo*. It then had a melting point of 116–117°.

Analysis—$C_{23}H_{33}O_3Br$. Calculated. C 63.15, H 7.61, Br 18.27
Found.   " 63.36, " 7.53, " 17.53

Pregnene-17-diol-3(α),21-one-11 Diacetate (XXVII)—A solution of 130 mg. of 21-bromopregnene-17-ol-3(α)-one-11 acetate (m.p. 113–115°) in 10 cc. of dry acetone[10] was refluxed with 800 mg. of anhydrous potassium acetate[10] for 5 hours; the potassium salts were then filtered off, the acetone removed *in vacuo*, and the residue taken up in ether. The ethereal solution was washed with dilute potassium carbonate, with water, and then

[9] The chloroform was washed seven times with equal volumes of water, dried over anhydrous sodium sulfate, and then distilled over phosphorus pentoxide.

[10] The acetone was twice distilled over fused potassium hydroxide. The potassium acetate was Merck's anhydrous, reagent grade, dried *in vacuo* at 180° for 6 hours.

concentrated to dryness on the steam bath. A small quantity of insoluble amorphous material was removed by extracting the residue with 30 cc. of warm petroleum ether (30–60°). The petroleum ether extract was concentrated to a small volume and cooled. The crystals so obtained melted at 112–113°. Recrystallization from acetone-petroleum ether and dilute acetone gave a pure product, m.p. 115.0–115.5°.

Analysis—$C_{25}H_{36}O_5$. Calculated, C 72.08, H 8.71; found, C 72.07, H 8.59

Pregnene-17-diol-3(α),21-one-11 (*XXVIII*)—A solution of 50 mg. of pregnene-17-diol-3(α),21-one-11 diacetate (XXVII) in 5 cc. of a 2 N solution of potassium hydroxide in 75 per cent dilute methanol was refluxed for 15 minutes. The addition of water to the cooled solution gave a crystalline precipitate, which after recrystallization from benzene or ethyl acetate melted sharply at 200.5°; $[\alpha]_D = +45.5°$.

Analysis—$C_{21}H_{32}O_3$. Calculated, C 75.85, H 9.70; found, C 75.98, H 9.35

Because of the difficultly crystalline nature of the 21-bromopregnene (XXVI) and pregnenediol diacetate (XXVII), appreciable quantities of material remained in the mother liquors of these two compounds. Further quantities of the pregnenediol (XXVIII) were obtained therefrom as follows: The mother liquors from the preparation of the 21-bromopregnene were combined and gave 1.90 gm. of a yellow oil. This was treated with potassium acetate and acetone as above. The product so obtained was extracted with petroleum ether and the latter was combined with the mother liquors from the preparation of the pregnenediol diacetate. The combined product was then saponified as before and extracted with ether. The partially crystalline residue weighed 1.45 gm. Direct crystallization of this from ethyl acetate and from acetone gave 420 mg. of crude pregnenediol (XXVIII), m.p. 194–197°, which was set aside. The 1.03 gm. of product contained in the mother liquors were treated with succinic anhydride in order to remove the more easily esterified 21-hydroxy compound. The crude product (1.03 gm.) was dissolved in 5 cc. of pyridine saturated at 28.4° with succinic anhydride. After 4 hours in a thermostat at 28.4°, the mixture was diluted with water and worked up as described below for the preparation of XXIX. The neutral fraction (750 mg.) was set aside for chromatography (see below), the acidic fraction was saponified with 2 N potassium hydroxide, and the neutral product obtained therefrom crystallized, giving 210 mg. of the crude pregnenediol, m.p. 192–196°. This was combined with the 420 mg. of crude diol obtained previously, and the whole was acetylated with pyridine and acetic anhydride and chromatographed. The resultant pure pregnenediol diacetate (XXVII) was saponified and the pure product so obtained was sufficient to bring the over-all yield of

XXVIII, based on the pregnene-20-diol-3(α),17(α)-one-11 monoacetate (XXV), to 49 per cent.

Pregnadiene-16,20-ol-3(α)-one-11 Acetate—This by-product from the reaction series XXV to XXVIII was obtained by chromatography of the neutral product remaining from the partial succinoylation described above. The 750 mg. were acetylated in the usual manner and the product (850 mg.) was taken up in 5 cc. of benzene and 100 cc. of petroleum ether. The solution was put on a column of 20 gm. of alumina and eluted with 100 cc. portions of petroleum ether-ether mixtures. The eluates obtained with 9:1, 8.75:1.25, and 8.5:1.5 petroleum ether-ether mixtures were crystallized from a small volume of methanol. Recrystallization from methanol and from petroleum ether by concentration gave small dense prisms, m.p. 127.0–129.5°; $\lambda_{max.} = 238$ mμ; $\epsilon = 1.50 \times 10^4$.

Analysis—$C_{23}H_{32}O_3$. Calculated, C 77.48, H 9.04; found, C 77.46, H 9.24

Pregnene-17-diol-3(α),21-one-11 Monohemisuccinate-21 (XXIX)—A solution of 1.16 gm. of pregnene-17-diol-3(α),21-one-11 in 5.5 cc. of pyridine previously saturated at 28.5° with succinic anhydride was allowed to stand for 120 minutes at 28.5° in a thermostat. Water was added to decompose the excess anhydride, and the mixture taken up in ether and washed with dilute hydrochloric acid. The organic acids were then removed by shaking the ethereal layer with a cold 10 per cent solution of potassium carbonate, which was then rendered acid to Congo red with dilute hydrochloric acid and extracted with ether. The washed ethereal solution of acids was evaporated and weighed 1.16 gm. The non-acidic ethereal solution after extraction with carbonate was washed and concentrated and unesterified diol recovered.

The 1.16 gm. of crude hemisuccinate were crystallized from ether and from dilute acetone, giving 800 mg. of product, m.p. 178.5–181.0°.

Analysis—$C_{25}H_{36}O_6$. Calculated, C 69.42, H 8.39; found, C 69.46, H 8.16

By saponification of the mother liquors of the hemisuccinate, recombining the diol obtained with that remaining unesterified from the previous succinoylation, and repetition of the partial esterification a nearly quantitative yield of the 21-monohemisuccinate was eventually obtained.

Pregnene-17-ol-21-dione-3,11 (XXX)—A solution of 1.47 gm. of pregnene-17-diol-3(α),21-one-11 monohemisuccinate-21 (XXIX) in 150 cc. of pure glacial acetic acid was cooled to 12° and treated dropwise with stirring with a solution of 700 mg. of chromic acid in 14 cc. of acetic acid and 14 cc. of water. The latter portions of the chromic acid solution were added more rapidly. The mixture was then allowed to warm to 17° over 60 minutes, the excess chromic acid destroyed with a small volume of

aqueous sodium sulfite, and the acetic acid removed *in vacuo*. The residue was taken up in ether, washed with water, and concentrated to dryness *in vacuo*. The colorless glass so obtained was heated on the steam bath for 1 hour with 5 cc. of 2 N aqueous sodium hydroxide together with 15 cc. of 10 per cent aqueous potassium carbonate. The solution was cooled, and the precipitated neutral product broken up into a powder, filtered, and washed with water. The dried product weighed 860 mg. The aqueous alkaline washings were acidified, extracted with ether, and the ether layer was concentrated to dryness, giving an acidic residue of 300 mg. This was saponified by refluxing for 15 minutes with 1.1 N methanolic potassium hydroxide. The solution was concentrated *in vacuo* to a small volume, diluted with water, and extracted with ether. The neutral fraction so obtained (185 mg.) was separated into ketonic (115 mg.) and non-ketonic fractions by the use of Girard's Reagent T, applied as described above. The 115 mg. of neutral ketonic material were combined with the 860 mg. of neutral fraction obtained from the previous saponification and the entire product was crystallized from ether-pentane. The crystals melted at 149–150° with preliminary sintering; $[\alpha]_D = +56°$; yield 81 per cent. Further recrystallization gave erratic results, with the melting point rising and falling irregularly. Consequently no further purification was attempted at this stage.

Pregnanetriol-17(β),20(β),21-dione-3,11 Diacetate-20,21 (XXXI)—Pregnene-17-ol-21-dione-3,11 (XXX) (850 mg.) was allowed to stand with a mixture of 7 cc. of pyridine and 7 cc. of acetic anhydride overnight. The solution was diluted with water, extracted with ether, washed successively with dilute hydrochloric acid, dilute alkali, and water, then evaporated to dryness. The 21-acetate was obtained as a colorless glass (910 mg.) which could not be obtained crystalline. After being thoroughly dried *in vacuo* at 100°, it was dissolved in 14 cc. of absolute ether, to which were then added 770 mg. of osmium tetraoxide and 450 mg. of dry pyridine. The solution began immediately to deposit brown amorphous osmate ester and subsequently some crystalline osmate-pyridine complex. After the solution had stood overnight, the ether was removed *in vacuo* and the residue refluxed for 3½ hours with a mixture of 35 cc. of alcohol, 24 cc. of water, and 5.5 gm. of sodium sulfite. The solution was then cooled to about 30° and filtered. The residue was extracted twice with 60 cc. portions of alcohol which were filtered. The combined filtrates were concentrated *in vacuo* to dryness, the residue was partly dissolved in 60 cc. of water, and the whole extracted thrice with 150 cc. portions of warm chloroform. The chloroform layer was separated, washed with a small volume of water, and concentrated to dryness *in vacuo*. The colorless gum weighed 910 mg. A small sample when triturated with water gave a product,

evidently a hydrate, which lost solvent of crystallization below 100° to give a non-crystalline gum again. In order to obtain a more readily purified derivative the crude triol was then converted to the diacetate by dissolving in a mixture of 5 cc. of pyridine and 5 cc. of acetic anhydride. After standing at room temperature for 40 hours, the mixture was treated with a small volume of water, concentrated *in vacuo*, and the residue taken up in chloroform, washed with dilute potassium carbonate, dilute hydrochloric acid, and water. The chloroform layer was concentrated to dryness *in vacuo* and the residue crystallized from ether. Recrystallization from a small volume of ethyl acetate gave needles, m.p. 212–213°; $[\alpha]_D$ = +93°. The mother liquors were chromatographed and gave additional diacetate. No other crystalline product was obtained; yield 86 per cent.

Analysis—$C_{25}H_{36}O_7$. Calculated. C 66.94, H 8.08
Found. " 67.58, 67.67, " 7.64, 7.38

The poor agreement of the analysis with theory is not readily explicable, since the sample for analysis was purified chromatographically and crystallized to constant melting point.

4 - Bromopregnanetriol-17(β),20(β),21-dione-3,11   Diacetate - 20,21 (XXXII)—To a solution of 126 mg. of pregnanetriol-17(β),20(β),21-dione-3,11 diacetate-20,21 in 0.50 cc. of acetic acid was added a solution of 42.8 mg. (0.95 mole) of bromine in 0.50 cc. of acetic acid. After a few minutes the bromine solution was suddenly decolorized and the mixture was poured into chloroform. The chloroform was washed with dilute sodium bicarbonate and water and concentrated to dryness *in vacuo*. The residue was crystallized from a small volume of absolute ether and melted at 188–189° with decomposition. The mother liquors were debrominated by stirring with acetic acid and zinc dust for a few minutes at room temperature. The recrystallized bromine-free product so obtained was rebrominated as before; yield 93 per cent.

Pregnene-4-triol-17(β),20(β),21-dione-3,11   Diacetate-20,21 (XXXIII) (Reichstein's Substance U Diacetate)—A solution of 710 mg. of 4-bromo-pregnanetriol-17(β),20(β),21-dione-3,11 diacetate-20,21 (XXXII) in 70 cc. of purified pyridine was refluxed for 10 hours. The pyridine was almost entirely removed *in vacuo*, and the residue dissolved in chloroform, washed with dilute hydrochloric acid, dilute potassium carbonate, water, and concentrated to dryness *in vacuo*. The 550 mg. of nearly colorless glass so obtained were dissolved in 50 cc. of benzene and absorbed on a column of 15 gm. of alumina prepared with petroleum ether. The column was eluted with 75 cc. of absolute ether, which gave traces of oil and crystals. The following ten eluates were obtained by passing through 50 cc. portions of ether-chloroform mixtures in the ratios 9:1, 8:2, 7:3, etc., down to un-

mixed chloroform. The successive fractions each weighed 50 to 55 mg. and melted in a steady downward progression from 246–249° to 204–208°. The lowest melting (last) fraction was recrystallized from methanol, combined with the next lowest melting fraction, recrystallized from methanol, and so on. By this method, 151 mg. of constant melting product were obtained. The mother liquors were again chromatographed, and the fractional crystallization repeated, giving an additional 130 mg. of pure product (46 per cent). The melting point was 252.0–253.5°; $[\alpha]_D = +179°$.[11] The ultraviolet absorption showed $\lambda_{max.} = 238.5$ mμ; $\epsilon = 2.83 \times 10^4$.

Analysis—$C_{25}H_{34}O_7$. Calculated, C 67.24, H 7.68; found, C 67.16, H 7.51

Pregnene-4-triol-17(β),20(β),21-dione-3,11 (Reichstein's Substance U) (I)—A solution of 260 mg. of pregnene-4-triol-17(β),20(β),21-dione-3,11 diacetate-20,21 (XXXIII) in 20 cc. of methanol was treated with 5 cc. of water containing 150 mg. of potassium carbonate and 250 mg. of potassium bicarbonate at 30°. The mixture was allowed to stand overnight at room temperature. It was then neutralized with 0.30 cc. of glacial acetic acid, concentrated *in vacuo* to a small volume, diluted with 100 cc. of water, and extracted with four 50 cc. portions of chloroform. The chloroform layer was washed wth a little aqueous potassium carbonate and then with water, the aqueous washings being reextracted each time with small portions of chloroform. The combined chloroform extract was concentrated to dryness *in vacuo* and the residue crystallized by triturating with a few drops of acetone. The crystals were washed with a mixture of cold ether-acetone, filtered, and recrystallized from ether-acetone. The mother liquors (35 mg.) were saponified and worked up as before. Total yield of pure material, 93 per cent, m.p. 208.5–209.5°;[12] $[\alpha]_D = +140°$.

Pregnene-4-triol-17(β),20(β),21-one-3 Monoacetate-21 (XXXVIII)—A sample of pregnene-4-triol-17(β),20(β),21-one-3 (210 mg.) was dissolved, with heating, in 0.50 cc. of absolute dioxane, and the solution cooled and treated with 77 mg. of dry pyridine and 84 mg. of acetic anhydride. After standing at room temperature overnight, the solution was treated with a small volume of water, concentrated *in vacuo* to dryness, and extracted with chloroform. The chloroform solution was washed with water and concentrated to dryness *in vacuo*. The residue was crystallized from ether and recrystallized from dilute alcohol. The melting point was then 189–192°. Mixed melting points with both the free triol and the 20,21-diacetate were about 145–175°. Mild saponification of the mother liquors gave back the free triol. The corrected yield was then 87 per cent.

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[11] Reichstein and von Euw (5) give 252–253° (corrected) and $[\alpha]_D = +178.5°$.

[12] Reichstein and von Euw (5) give 208° (corrected); no rotation is given.

Analysis—$C_{21}H_{31}O_4CH_3CO$. Calculated. C 70.73, H 8.78, CH_3CO 11.0
                                    Found.   " 70.73, " 9.07,   "   10.6

Pregnene-4-diol-17(β),21-dione-3,20 Monoacetate-21 (Reichstein's Substance S Monoacetate) (XXXIX) and Androstene-4-dione-3,17 (XL)—A solution of 200 mg. of pregnene-4-triol-17(β),20(β),21-one-3 monoacetate-21 (XXXVIII) in 18 cc. of pure redistilled glacial acetic acid was cooled to 11° and treated dropwise over 10 minutes with a solution of 100 mg. of chromic acid in 2 cc. of water. The solution was then kept at 11° for 1 hour and the excess chromic acid destroyed by the addition of a small volume of dilute sodium sulfite. The acetic acid was removed *in vacuo* (bath temperature 55°), and the residue taken up in chloroform, washed with water, dilute potassium carbonate, again with water, and concentrated to dryness *in vacuo*. The residue weighed 192 mg. It was taken up in 10 cc. of benzene and put over a column of 6 gm. of alumina. Mixtures of ether-petroleum ether were then used to elute the product. From a 3:7 mixture of petroleum ether-ether mixture to absolute ether alone was obtained a total of 43 mg. of crystals; m.p., after recrystallization from acetone-petroleum ether, 171.5–172°. Admixture with an authentic sample of androstene-4-dione-3,17 gave no depression. Successive elutions with ether-chloroform mixtures from 9:1 down to 6:4 ether-chloroform gave crude crystals which melted at 224–238° (9:1 fraction) down to 206–225° (6:4 fraction). These fractions were combined and weighed 36.5 mg. After two recrystallizations from methanol and one from acetone-petroleum ether, a product[13] was obtained which had a melting point of 235–238° (corrected) with slight sintering at 232°; $[\alpha]_D = +118° \pm 4°$ $(c = 0.7)$; $\lambda_{max.} = 242\ m\mu$; $\epsilon = 1.47 \times 10^4$. The compound (XXXIX) reduced an ammoniacal solution of silver nitrate strongly and rapidly at room temperature and gave the characteristic scarlet color with concentrated sulfuric acid. The yield was 13 mg.

Analysis—$C_{23}H_{32}O_5$. Calculated, C 71.10, H 8.30; found, C 71.08, H 8.33

The fractions obtained by subsequent elution of the column consisted chiefly of starting material (m.p. 190–196°, corrected) and a small amount of 20,21-diacetate which doubtless was originally present in the starting material.

Pregnene-4-triol-17(β),20(β),21-dione-3,11 Monoacetate-21 (XXXIV)—A suspenson of 179.5 mg. of pregnene-4-triol-17(β),20(β),21-dione-3,11 in 0.50 cc. of absolute dioxane was heated on the steam bath until nearly all of the crystals dissolved. The solution was then rapidly cooled to room temperature and treated with a mixture of 63.5 mg. of pyridine and 68.5

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[13] Reichstein and von Euw (25) give 239–241°, 236–238°, and 235–237° (corrected) as the melting points of various samples of this compound; $[\alpha]_D = +116.33° \pm 4°$.

mg. of acetic anhydride. After standing at room temperature overnight, the mixture was treated with 5 cc. of water and the solvent removed *in vacuo*. This was repeated with another portion of water and then with two 5 cc. portions of chloroform. The colorless glass so obtained was dissolved in 0.5 cc. of chloroform and 15 cc. of benzene and chromatographed over 6.4 gm. of alumina. The fractions from 1:1 ether-chloroform to 2:8 ether-chloroform consisted of diacetate; from 1:9 ether-chloroform to 7:3 chloroform-acetone crystals were obtained by rubbing the concentrated eluates with ether. The bulk of these fractions melted at 172–174°. The material could be recrystallized, with some difficulty, from acetone-ether. The melting point was unaltered; yield (of all material, m.p. about 169–174°) 61 per cent. All of the remaining chromatographic fractions were saponified and the triol so obtained was reacetylated (79 mg. of triol, 0.22 cc. of dioxane, 19.8 mg. of acetic anhydride, and 18.7 mg. of pyridine in 0.23 cc. of dioxane; room temperature for 40 hours). Chromatography of the product gave additional monoacetate; total yield 87 per cent.

Analysis—$C_{23}H_{32}O_6$. Calculated, C 68.29, H 8.00; found, C 67.90, H 7.92.

Pregnene-4-diol-17(β),21-trione-3,11,20 Monoacetate-21 (Kendall's Compound E Monoacetate) (XXXVI) and Adrenosterone (XXXV)—To a solution of 165 mg. of pregnene-4-triol-17(β),20(β),21-dione-3,11 monoacetate-21 (XXXIV) in a mixture of 13 cc. of glacial acetic acid and 2 cc. of water at 11° was added dropwise, with stirring, a solution of 85 mg. of chromium trioxide in 1.7 cc. of water. After standing at 11° for 15 minutes, the solution was warmed to 24° and left at that temperature for 75 minutes. A dilute solution of sodium sulfite was added dropwise to destroy the excess chromium trioxide, the acetic acid then concentrated to a small volume *in vacuo*, and the residue taken up in chloroform. The chloroform layer was washed with dilute potassium carbonate, then with water, and concentrated to dryness *in vacuo*. The residue was taken up in 10 cc. of benzene and put on a column of 4.5 gm. of alumina prepared with petroleum ether. Fractional elution with successive 15 cc. portions of solvents then gave the results presented in Table I.

Fractions XVIII to XXI consisted essentially of unoxidized starting material; this material was combined and reoxidized as before except that no water other than that contained in the chromic acid solution was added; the oxidation mixture then stood at 11° for 90 minutes rather than at room temperature. The product was worked up and chromatographed as before. Fraction XII from the first chromatogram was added to the second oxidation product before chromatographing.

Fractions IV to XI represented crude adrenosterone and were combined with the comparable fractions from the second chromatogram. Total

crude fraction, 54 mg. Recrystallization from acetone-petroleum ether and from methanol gave 35 mg. of constant melting product, m.p. 222–224°; $[\alpha]_D^{25} = +281°$ (acetone);[14] $\lambda_{max.} = 239$ mμ; $\epsilon = 1.38 \times 10^4$.

Analysis—$C_{19}H_{24}O_2$. Calculated, C 75.97, H 8.05; found, C 75.83, H 8.13

Fractions XIV and XV were combined with the corresponding fractions from the second chromatogram; this material represented crude Compound

TABLE I

Chromatographic Separation of Compound E ◆

Fraction No.	Solvent	Weight	M.p.
		mg.	*°C.*
I	7.5:7.5 petroleum ether-ether		
II	6.0:9.0 " "	Trace, crystals	
III	4.5:10.5 " "	" oil	
IV	3.0:12.0 " "	2.7 crystals	220–224
V	1.5:13.5 " "	5.0 "	210–213
VI	Ether	8.5 "	
VII	14:1 ether-chloroform	9.0 "	224–227
VIII	13:2 "	7.0 "	222–225
IX	12:3 "	5.4 "	225–226.5
X	11:4 "	2.4 "	
XI	10:5 "	2.0 "	
XII	9:6 "	3.5 "	211–220
XIII	8:7 "	3.5 "	235–241
XIV	7:8 "	1.5 "	219–235
XV	6:9 "	5.0 " cubes	221–230
		" needles	231–248
XVI	5:10 "	4.0 "	200–220
XVII	4:11 "	5.0 "	200–240
XVIII	3:12 "	9.0 "	140–160
XIX	2:13 "	9.0 "	
XX	Pure chloroform	13.0 "	
XXI	10:10 chloroform-acetone	50.0 "	
		145.5 "	

E acetate (XXXVI). The quantity of the product was increased by combining Fractions XV and XVI with the comparable fractions from the second chromatogram and rechromatographing. Total weight of the mixture, 18.7 mg. consisting of XXXVI and probably the diacetate (XXXIII). The 18.7 mg. were dissolved in a small quantity of benzene, put on a column of 0.60 gm. of alumina, and eluted with 4 cc. portions of ether-chloroform mixtures (see Table II).

[14] Reichstein gives the melting point as 222–224° (3); $[\alpha]_D$ $+262°$ (alcohol) (26) for a sample isolated from natural sources.

Fractions III to VI were combined with the crude Compound E acetate obtained from the two previous chromatograms (total, 29.6 mg.) and recrystallized from acetone-ether, from methanol, and finally from a small volume of acetone. It then had a melting point[15] of 235–238° with slight sintering at 230°; $[\alpha]_D = +164° \pm 4°$ ($c = 0.5$); $\lambda_{max.} = 238$ mμ; $\epsilon = 1.58 \times 10^4$. It reduced a solution of ammoniacal silver nitrate strongly at room temperature. An authentic sample of Kendall's Compound E monoacetate, which had been purified chromatographically, had on our apparatus a melting point of 235–240°, with slight sintering at 225°.

A mixed melting point with the synthetic sample showed no depression. An absorption spectrum of the authentic compound showed $\lambda_{max.} = 238$ mμ; $\epsilon = 1.59 \times 10^4$. In general, the curves were completely identical within experimental error.

Analysis—$C_{23}H_{30}O_6$. Calculated, C 68.61, H 7.52; found, C 68.52, H 7.23

TABLE II

Chromatographic Separation of Compound E

Fraction No.	Solvent	M.p.
		°C.
I	7:1 ether-chloroform	Trace, oil
II	6:2 "	Poorly crystalline
III	5:3 "	222–238
IV	4:4 "	212–233
V	3:5 "	215–230
VI	2:6 "	215–225
VII	1:7 "	Poorly crystalline
VIII	Chloroform	Oil

SUMMARY

The partial synthesis of $\Delta^{4,5}{}_2$-pregnene-4-diol-17(β),21-trione-3,11,20 acetate (Kendall's Compound E acetate) from desoxycholic acid is described. Other naturally occurring adrenal steroid compounds obtained as intermediates or by-products of the synthesis are $\Delta^{4,5}{}_2$-pregnene-4-triol-17-(β),20(β),21-dione-3,11 (Reichstein's Substance U) and adrenosterone.

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[15] Reichstein (27) gives a melting point of 239–241° (corrected) for the acetate of his Substance Fa, isolated from natural sources; no rotation is given.

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IMPROVED METHODS FOR THE PREPARATION OF
3(α)-HYDROXY-Δ⁹,¹¹-CHOLENIC ACID*

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Any method for the synthesis of Compound E (1) must involve the introduction of oxygen at the C_{11} position, either as a secondary hydroxyl

(I)

or as a keto group. Of all the approaches to such a synthesis afforded by general methods of organic chemistry, the addition reactions of a double bond, in this case at either C_9–C_{11} or at C_{11}–C_{12}, are the most attractive. The fact that procedures for synthesizing both of these unsaturated compounds from desoxycholic acid have been published (1–3) provides sources of starting material. However, the low yields reported in these preparations have led to attempts to modify the procedures, thereby increasing the yield of the desired products. This study deals with the preparation of the compounds containing the double bond at the C_9–C_{11} position.

The work of Chakravorty and Wallis (1) on the preparation of 3(α)-hydroxy-Δ⁹,¹¹-cholenic acid provided for the first time a method for introduction of the double bond at the C_{11} position. This was accomplished in the following manner. 3(α)-Acetoxy-12-ketocholanic acid was brominated with 1.05 moles of bromine at 70° for 4 hours to give the corresponding 11-bromo derivative. This compound was dehydrobrominated to 3(α)-hydroxy-Δ⁹,¹¹-12-ketocholenic acid by refluxing for 2 hours with 10 per cent sodium ethylate solution. The semicarbazone was
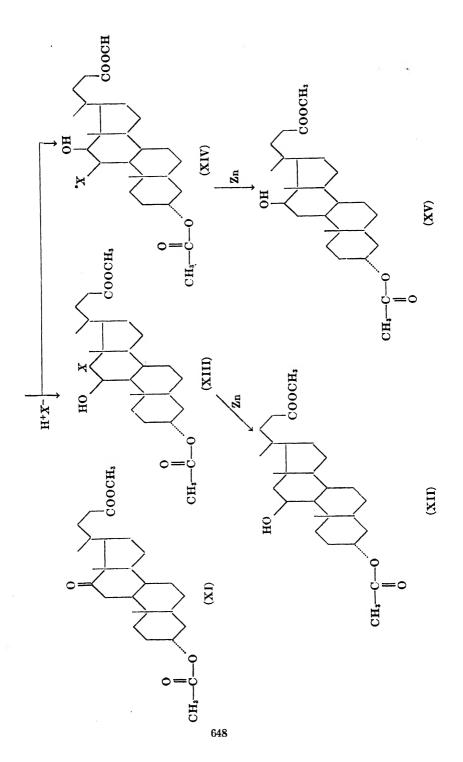
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prepared by refluxing for 2 hours with semicarbazide acetate in dilute alcohol. This was reduced by heating with sodium ethylate for 15 hours at 180° to give the desired hydroxycholenic acid of melting point 183–184°. The over-all yield, however, was poor and in this paper we wish to record certain improvements. Each of the above reactions has been restudied for the purpose of obtaining optimum conditions for maximum yields of the desired 3(α)-hydroxy-$\Delta^{9,11}$-cholenic acid. The results of these experiments are described below.

Preparation of the C_{12} Ketone—The method of Schwenk and collaborators (4) for the direct oxidation of 3(α)-succinoxy-12-hydroxycholanic acid was employed. A mixture of succinic anhydride, pyridine, and desoxycholic acid was heated for 3 hours on the steam bath; the reaction was nearly quantitative and the subsequent oxidation at room temperature resulted in the preparation of the crystalline C_{12}-keto compound in excellent yields. Alkaline saponification led to pure 3(α)-hydroxy-12-ketocholanic acid, its physical properties and conversion to various derivatives indicating excellent purity.

α-Bromination of the C_{12} Ketone—The high temperature bromination employed by Chakravorty and Wallis (1) involves the addition in one portion of 1.05 moles of bromine in acetic acid to a solution of 3(α)-acetoxy-12-ketocholanic acid in the presence of a small amount of hydrobromic acid. In a restudy of this reaction, we have found that maintaining the solution at 70° for 4 hours reduces the yield of desired product and leads to a mixture of by-products produced by halogenation reactions other than at C_{11}. Therefore, experiments were made under varying conditions with 3(α)-formoxy, 3(α)-acetoxy, and 3(α)-succinoxy compounds, and with the methyl ester of 3(α)-benzoxy-12-ketocholanic acid.

With pure crystalline 3(α)-formoxy-12-ketocholanic acid it was found that although bromination at room temperature favors the formation of halogen-substituted derivatives the compound brominates in an anomalous manner to give a crystalline tribromo derivative.

Bromination of 3(α)-succinoxy-12-ketocholanic acid was found to require a temperature of 90°. The product was a crystalline material melting at 234° with decomposition and having, on analysis, 1 bromine atom per molecule. Unfortunately, owing to difficulties in dehalogenation, the bromination of the succinate could not be used as a preparative method.

Our next experiments were conducted on the acetoxy derivative. When bromination of 3(α)-acetoxy-12-ketocholanic acid was carried out at 55° over a period of 6 hours (5), a clear colorless product was obtained, which on reesterification could be isolated in crystalline form.

Good results were also obtained by the rapid bromination of methyl

$3(\alpha)$-benzoxy-12-ketocholanate at 90°. Although in this case no crystalline product was isolated, the non-crystalline monobromo compound was produced in a high yield. Thus, despite the fact that it was necessary to use the increased temperature to effect the halogenation, various side reactions observed with other derivatives did not appear to take place.

Ethylate Dehydrohalogenation of C_{11}-Bromo-12-ketocholanic Acid Derivatives—In their studies on the reaction of sodium ethylate on $3(\alpha)$-acetoxy-11-bromo-12-ketocholanic acid the time of refluxing used by Chakravorty and Wallis (1) was 2 hours. Because of the low yield obtained by this method, it was suspected that since α,β-unsaturated ketones are often unstable in alkali, the refluxing time used by these workers resulted in some decomposition in Ring C of the steroid nucleus. Therefore experiments were carried out with shorter periods of dehydrohalogenation, the time of refluxing varying from 5 to 30 minutes. The results obtained indicate that, although in every case the reaction approaches completion, it is necessary to reflux with ethylate only 15 minutes to render the per cent of unchanged bromo compound negligible; consequently shorter periods of dehydrohalogenation were employed.

The detailed results obtained are described in the experimental part of this paper. However, it is pertinent to summarize briefly certain of these observations. Dehydrohalogenation of the bromination product of $3(\alpha)$-formoxy-12-ketocholanic acid resulted in a mixture which for the most part appeared to be composed of the unsaturated cholenic acid and $3(\alpha)$-hydroxy-12-ketocholanic acid. Only a small amount of the pure $3(\alpha)$-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid could be obtained by crystallization because of its similarity in solubility to the saturated compound. Under the same conditions it was found that the brominated succinoxy derivative gave very unsatisfactory results; the ethylate reduction of this compound proceeded violently, and the product was no more pure than in the case of the formoxy derivative.

In sharp contrast to these first two derivatives, which give unsatisfactory dehydrohalogenation products even under the milder conditions developed, stands the observation that the $3(\alpha)$-benzoxy and $3(\alpha)$-acetoxy compounds yield products of the highest purity. In these two cases after a single recrystallization, pure $3(\alpha)$-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid is obtained in 25 per cent yield, and sufficient material is obtained in further crops to increase the yield to 40 per cent. Further increased yields of the desired $\Delta^{9,11}$-unsaturated 12-keto compound were observed when the mother liquors from the above crystallizations were dissolved in ether and treated with gaseous hydrogen bromide. The addition product so obtained was insoluble in ether and easily separated. On solution in warm acetic acid the compound lost hydrogen bromide and on cooling gave $3(\alpha)$-hydroxy-

$\Delta^{9,11}$-12-ketocholenic acid. In this manner the over-all yield based upon 3(α)-hydroxy-12-ketocholanic acid was increased to 65 per cent.

In addition it was found that formic acid treatment of the mother liquors from these two reactions enables the recovery of about 35 per cent of the saturated C_{12}-keto compound. By this method, therefore, the yield of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid was greatly increased. Its high degree of purity is indicated by its extinction coefficient in absorption spectra measurements.

Wolff-Kishner Reduction of the α,β-Unsaturated Ketone—Preparation of the semicarbazone of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid (II) prior to ethylate reduction was effected simply by refluxing a solution of the ketone and semicarbazide in alcohol. It was found that increasing the time of heating of the reaction mixture from the 2 hour period used by Chakravorty and Wallis (1) to the more generally employed 8 hour period results in a quantitative reaction. The crystalline product melts with decomposition at 218°.

It should be noted in conclusion that the original authors (1) had effected the Wolff-Kishner reduction of the above semicarbazone in fair yields by reaction in a sealed tube at 180° for 16 hours. In our hands these yields, as well as the purity of the resulting compound, improved by decreasing

(II)   (III)

(IV)

the time of heating in the autoclave to 8 hours. Under such conditions a 70 per cent yield of crude product melting at 176° can be obtained as needles. Two recrystallizations from acetone yielded a highly pure product of melting point 184° (III). Acetylation and methylation resulted in crystalline plates of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate (IV) melting at 135°; $[\alpha]_D^{23} = +63°$.

It can thus be seen that the preparation of pure 3(α)-hydroxy-$\Delta^{9,11}$-cholenic acid from desoxycholic acid may be accomplished in yields varying from 30 to 50 per cent.

<div align="center">EXPERIMENTAL</div>

3(α)-Succinoxy-12-hydroxycholanic Acid—This compound was prepared by the method of Schwenk, Riegel, Moffett, and Stahl (4). To 500 cc. of dry pyridine were added 200 gm. of pure desoxycholic acid and 200 gm. of succinic anhydride. After being refluxed for 6 hours, during which time the solution turned black, the contents of the flask were poured into water. The tan-colored suspension was extracted with ether, washed four times with hydrochloric acid (1:5), twice with water, and dried. Upon removal of the ether by distillation a crystalline residue was obtained which melted at 224.5–226°. Yield, 236 gm. Recrystallization from alcohol gave a product which melted at 227° and agreed in other properties with the same succinoxy derivative described by Schwenk *et al.* (4).

3(α)-Succinoxy-12-ketocholanic Acid—A portion (58.4 gm.) of 3(α)-succinoxy-12-hydroxycholanic acid prepared by the method described above was dissolved in 610 cc. of glacial acetic acid and treated at 15° during the course of 45 minutes with 14 gm. of chromic acid dissolved in 175 cc. of 90 per cent acetic acid. Stirring was continued for another 45 minutes after the chromic acid had been added. The resulting 57.2 gm. of crude crystalline material melted at 234–238°. Recrystallization from alcohol gave a pure product of melting point 236–238°, identical in properties with the same compound prepared by Schwenk *et al.* by a similar method.

3(α)-Hydroxy-12-ketocholanic Acid—10 gm. of 3(α)-succinoxy-12-ketocholanic acid were saponified for 1 hour on the steam bath with 6.6 gm. of potassium hydroxide in 50 cc. of water. The mixture was poured into an ice-acid mixture; the white precipitate when washed and dried weighed 7.9 gm. and melted unsharply at 157–159°. Recrystallization from ethyl acetate gave needles melting at 159–161.5°. Esterification with a 1 per cent solution of hydrogen chloride in methanol gave the methyl ester in 97 per cent yield. Acetylation of the methyl ester with acetic anhydride in pyridine gave methyl 3(α)-acetoxy-12-ketocholanate, m.p. 148.5–150.5°. $[\alpha]_D^{27} = +107.5°$ (57.2 mg. in 5 cc. of absolute alcohol solution). The over-all yield from desoxycholic acid was 86 per cent.

3(α)-Hydroxy-$\Delta^{9,11}$-12-ketocholenic Acid.   From 3(α)-Succinoxy-11-bromo-12-ketocholanic Acid—10 gm. of 3(α)-succinoxy-12-ketocholanic acid were dissolved in 25.6 cc. of glacial acetic acid and treated with 25.6 cc. of a 1.05 M bromine-acetic acid solution.   The reaction was allowed to continue for 1 hour, after which time most of the color had disappeared.   The resulting crystalline monobromide melted at 234°.   4 gm. of this material dissolved in 20 cc. of hot absolute alcohol were added carefully to a boiling solution of sodium ethylate prepared from 4 gm. of sodium and 47.5 cc. of absolute alcohol.   As the reaction generally was not satisfactory and very little sodium bromide was liberated, the mixture was refluxed for 10 minutes.   When the product was worked up in the usual manner, the residual oil on crystallization from ethyl acetate gave low yields of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid of melting point 158–161°.   Recrystallization gave crystals melting at 160–163°, compared with 171° for the pure compound first prepared by Chakravorty and Wallis (1).

From Methyl 3(α)-Benzoxy-11-bromo-12-ketocholanate—100 gm. of methyl 3(α)-hydroxy-12-ketocholanate in 500 cc. of benzene were treated with 24.4 cc. of benzoyl chloride dissolved in 50 cc. of dry pyridine.   After completion of the reaction 92 gm. of methyl 3(α)-benzoxy-12-ketocholanate of melting point 94–95° were obtained.   20 gm. of this product in 54 cc. of glacial acetic acid were brominated with 54 cc. of 1.05 M bromine-acetic acid solution at 85° for 45 minutes.   The resulting 20 gm. of oily product were dissolved in 100 cc. of hot absolute alcohol and added slowly to a boiling solution of sodium ethylate (20 gm. of sodium in 240 cc. of absolute alcohol).   The reaction proceeded smoothly, giving sodium bromide immediately.   Water was added after 15 minutes of refluxing and the product worked up.   Two recrystallizations from ethyl acetate gave 4.9 gm. of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid of melting point 172–173°. $[\alpha]_D^{20} = +113°$ (15.4 mg. in 2 cc. of absolute alcohol).   Further crops of crystals increased the yield.

From 3(α)-Acetoxy-11-bromo-12-ketocholanic Acid—A solution of 90 gm. of 3(α)-hydroxy-12-ketocholanic acid in 200 cc. of dry pyridine and 200 cc. of acetic anhydride was heated for 18 hours on the water bath.   The amorphous product on crystallization from aqueous acetic acid gave 63 gm. of material melting at 192°.   From the mother liquor additional amounts of the acetoxy derivative were obtained.   To 60 gm. of this compound dissolved in 600 cc. of glacial acetic acid at 50–60° was added over a period of 3 hours a solution of 12 cc. of bromine in 480 cc. of acetic acid.   After standing 4 more hours at 55° it was worked up in the usual manner to yield a non-crystalline bromo derivative.   20 gm. of this product dissolved in 130 cc. of absolute alcohol were added slowly to a refluxing solution of sodium ethylate (24 gm. of sodium in 275 cc. of absolute alcohol).

The reaction proceeded smoothly and after 10 minutes the product was worked up. Crystallization from ether and from ethyl acetate gave 4 gm. of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid which melted at 168–170°. From the mother liquor additional amounts of this compound were obtained to increase the yield to 65 per cent. In one case this was accomplished by dissolving in ether the material in the mother liquors and treating the solution with gaseous hydrogen bromide. The addition product so obtained was crystallized and filtered. On solution in warm acetic acid the compound lost hydrogen bromide and on cooling additional amounts of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid crystallized.

From 3(α)-Formoxy-11-bromo-12-ketocholanic Acid—When 7.9 gm. of 3(α)-hydroxy-12-ketocholanic acid were dissolved in 25 cc. of 98 per cent formic acid and the solution allowed to stand for 12 hours, a crystalline 3(α)-formoxy derivative in 97 per cent yield was obtained, m.p. 203–204°. Recrystallization from alcohol gave 7 gm. of product, m.p. 203–204.5°, $[\alpha]_D^{17} = +116.5°$ (17.3 mg. in 2 cc. of absolute alcohol). A solution of 25 gm. of this product in 75 cc. of glacial acetic acid was treated for 16 hours at room temperature with 75 cc. of a 1.05°M bromine-acetic acid solution. The product was then worked up in the usual manner. From a concentrated ether solution (250 cc.) a small amount of crystalline material separated. Weight, 2 gm.; m.p. 135–142° (with decomposition). Recrystallization from alcohol gave crystals which melted at 145.5–147°, $[\alpha]_D^{20} = +24.2°$ (49.4 mg. in 5 cc. of acetone solution).

Analysis—$C_{25}H_{35}O_5Br_3$. Calculated, C 45.80, H 5.39; found, C 46.20, H 5.58

The ether filtrate was evaporated to dryness to give a brominated non-crystalline residue. A solution of 3.6 gm. of this material in 20 cc. of absolute alcohol was poured slowly into a refluxing sodium ethylate solution (4 gm. of sodium dissolved in 46 cc. of absolute alcohol). Refluxing was continued for 15 minutes, after which the product was worked up. Great difficulty was encountered in getting a crystalline material of high purity. The first recrystallization from ether gave a product which melted unsharply at 158–163°. Five recrystallizations from ethyl acetate gave 0.5 gm. of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid, m.p. 168–169°. The 3(α)-formoxy derivative was prepared by dissolving a portion of this material in 98 per cent formic acid. Recrystallization from dilute acetone gave white plates which melted at 169–170°.

3(α)-Hydroxy-$\Delta^{9,11}$-cholenic Acid—To 15.7 gm. of pure 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid dissolved in 120 cc. of alcohol were added 9.5 gm. of sodium acetate and 7.8 gm. of semicarbazide hydrochloride in 15 cc. of water. The solution was refluxed for 8 hours and the alcohol was removed by distillation. The crystalline residue was leached with hot

water, dried, and repeatedly washed with ether. The product melted at 218–221° and weighed 15 gm. To 2 gm. of this semicarbazone was added a solution of sodium ethylate (6 gm. of sodium in 70 cc. of absolute alcohol) and the contents were sealed in a tube and placed in an oven at 180° for 12 hours. The material was worked up and 0.9 gm. of crude product, m.p. 173–178°, was obtained. This yield was increased to 70 per cent when the time of heating at 180° was cut to 8 hours. Four recrystallizations from acetone gave plates which melted at 181–184°. $[\alpha]_D^{20} = +54.2°$ (15.5 mg. in 2 cc. of absolute alcohol solution).

The crystalline acetoxy methyl ester was prepared in excellent yield by the action of diazomethane followed by acetylation. It melted at 133–135° and had $[\alpha]_D^{23} = +62.9°$ (92.4 mg. in 10 cc. of absolute alcohol).

Methyl 3(α)-Hydroxy-$\Delta^{9,11}$-12-ketocholenate—Methylation of 1.0 gm. of 3(α)-hydroxy-$\Delta^{9,11}$-12-ketocholenic acid with 0.34 gm. of diazomethane in ether gave an oil which, on crystallization from ether, melted at 116–117°. $[\alpha]_D^{22} = +109.5°$ (21.0 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{25}H_{39}O_4$. Calculated, C 74.54, H 9.51; found, C 74.63, H 9.53

3(α)-Acetoxy-$\Delta^{9,11}$-12-ketocholenate—1 gm. of the methyl ester prepared by the method described above was treated on the water bath with 5 cc. of acetic anhydride in 10 cc. of dry pyridine. When the product was worked up, an oil was obtained which, on crystallization from methanol, gave crystals of melting point 138–139°; $[\alpha]_D^{18} = +110.8°$ (22.4 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{27}H_{40}O_5$. Calculated, C 72.94, H 9.07; found, C 72.90, H 9.17

SUMMARY

Improved methods have been developed by which the preparation of 3(α)-hydroxy-$\Delta^{9,11}$-cholenic acid from desoxycholic acid may be effected in yields of 50 per cent.

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THE INTRODUCTION OF OXYGEN INTO THE STEROID NUCLEUS AT THE C_{11} POSITION*

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Concurrently with studies made on the preparation and rearrangement of oxides (1) derived from C_9–C_{11}- and C_{11}–C_{12}-unsaturated steroids, other addition reactions to these double bonds have been studied. In this paper we wish to describe our results obtained by the action of hypobromous acid on methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate. Application was made of the Schmidt reaction of hypobromous acid with double bonds. With methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate (I) a crystalline product (II) was obtained. Chromic acid oxidation of this latter compound followed by debromination with zinc in acetic acid yielded an oil which partially crystallized. Chromatographic fractionation yielded two products. Fraction I melted at 132° and was found to be identical with the starting mate-

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rial (I).   Fraction II melted at 132–134°, with sintering at 118°.   This compound we believe to be methyl 3(α)-acetoxy-11-ketocholanate (III). A mixed melting point determination with a product prepared according to the method of Ott and Reichstein (2) by application of the Schmidt reaction to methyl 3(α)-acetoxy-Δ^{11}-cholenate, and ascribed by them the same structure, gave no depression.

In this connection we should like to point out that the action of nitrosyl chloride on methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate has also been studied. Pure nitrosyl chloride prepared according to the method of Skinner (3) was allowed to react with this unsaturated steroid at room temperature for 20 hours.   Removal of the solvent in the cold yielded a crystalline, colorless material which began to decompose at 10°.   Because of the instability of this compound all attempts to remove halogen and obtain the desired ketone either as such or in the form of its oxime failed.   In our hands only starting material was isolated.

<div style="text-align:center">EXPERIMENTAL</div>

Methyl 3(α)-Acetoxy-11-ketocholanate.   From Methyl 3(α)-Acetoxy-$\Delta^{9,11}$-cholenate—To a solution of 500 mg. of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate dissolved in 15 cc. of tertiary butyl alcohol and 6 cc. of 0.4 N sulfuric acid were added 220 mg. of N-bromoacetamide.   After 2 minutes a bright yellow color developed in the solution.   The reaction was stopped and sodium bisulfite solution was added until decolorization was complete. Water was added slowly and on standing a crystalline material separated. This product was filtered and dried.   It melted at 131–133° and was found to be starting material.   The mother liquors were concentrated to dryness.

A solution of 200 mg. of this material dissolved in 5 cc. of glacial acetic acid was treated for 12 hours at room temperature with 0.10 gm. of chromic acid dissolved in 1 cc. of acetic acid.   The mixture was then poured into water, extracted with ether, washed with carbonate solution, and with water, and dried.   On evaporation a crude crystalline product was obtained which gave a positive Beilstein halogen test.

To a solution of 170 mg. of this crude bromoketone in 5 cc. of acetic acid was added an excess of zinc dust.   The mixture was stirred on the steam bath for 25 minutes.   Filtration of the zinc was followed by removal of the acetic acid.   The residue was taken up in ether, washed with sodium carbonate solution and water, and dried.   On evaporation 150 mg. of an oily product were obtained.   This material was fractionated chromatographically as follows: It was dissolved in 20 cc. of petroleum ether (30–60°)-benzene solution (1:2), and placed on a column of 8 gm. of acid-washed Merck's alumina.   The pressure head was 220 mm. of mercury.

On developing the chromatogram and elution with benzene-petroleum ether (30–60°) in varying proportions (10 per cent benzene to 70 per cent benzene), two crystalline fractions were obtained. Fraction I weighed 37 mg. and on recrystallization melted at 132°. A mixed melting point determination with methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate gave no depression.

Fraction II weighed 40 mg., and recrystallization of a portion of this material from methanol gave plates which melted at 132–134°, with sintering at 118°. $[\alpha]_D^{19} = +68.5°$ (20 mg. in 2 cc. of acetone solution). A mixed melting point determination with 3(α)-acetoxy-$\Delta^{9,11}$-cholenate, m.p. 131–134°, gave a depression. A mixed melting point determination with a sample of methyl 3(α)-acetoxy-11-ketocholanate of melting point 130–133° (sintering at 118°), prepared from methyl 3(α)-acetoxy-Δ^{11}-cholenate according to the method of Ott and Reichstein (2), gave no depression.

Action of Nitrosyl Chloride on Methyl 3(α)-Acetoxy-$\Delta^{9,11}$-cholenate—To 225 mg. of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate (m.p. 131–134°) dissolved in 20 cc. of anhydrous ether was added 0.10 cc. of pure liquid nitrosyl chloride prepared according to the method of Skinner (3). A glass stopper was inserted in the flask and the solution was allowed to stand at room temperature for 20 hours. After this time the solution was cooled to 0° and the liquid removed *in vacuo*. As the solution was concentrated, clear crystals formed which gave a very strong positive Beilstein test. When the flask was allowed to warm up to about 10°, decomposition of the crystalline product began. This was stopped immediately by cooling and by dissolving the product in 25 cc. of dry ether.

To this solution were added 50 mg. of Adams' palladium oxide catalyst and the contents were submitted to hydrogenation under 1 atmosphere of hydrogen. The solution was shaken overnight at room temperature, and after filtration of the catalyst, it was evaporated *in vacuo* at 0°.

To insure complete removal of halogen the oily product as obtained was dissolved in 15 cc. of acetic acid and treated with zinc dust. When the product was worked up, an oil was isolated which was reesterified and reacetylated. After chromatographic fractionation 66 mg. of a crystalline product were obtained which, when recrystallized from methanol, melted at 129–132°. A mixed melting point determination with a sample of methyl 3(α)-acetoxy-11-ketocholanate gave a depression. When a similar mixed melting point determination was made with a sample of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate, no depression was observed.

SUMMARY

A method is described for the introduction of oxygen into the steroid nucleus at the C_{11} position.

Methyl $3(\alpha)$-acetoxy-11-ketocholanate has been prepared from methyl $3(\alpha)$-acetoxy-$\Delta^{9,11}$-cholenate by the action of hypobromous acid, followed by oxidation of the secondary hydroxyl group and by reductive removal of the halogen. The compound crystallizes from methanol in large flat hexagonal plates of melting point 132–134° (sintering at 118°); $[\alpha]_D^{19} = +68.5°$ (acetone).

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STUDIES ON THE PREPARATION AND REARRANGEMENT
OF CERTAIN STEROID OXIDES*

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By preparative methods developed by Chakravorty and Wallis (1) and improved by Hicks, Berg, and Wallis (2) and by Alther and Reichstein (3) the 3(α)-hydroxycholenic acids containing double bonds at the C_9–C_{11} and the C_{11}–C_{12} positions respectively are readily available. An attractive method, therefore, for introducing oxygen either as a ketone or as a secondary hydroxyl at the C_{11} position lies in addition reactions of these unsaturated compounds. It would seem that one such method of introduction of this grouping essential in the synthesis of Compound E would consist in the rearrangement of oxides derived from the two cholenic acids mentioned above. Such reactions might be imagined to proceed according to the accompanying diagrams (I to XV).

If instead of rearrangement simple hydrolysis of the oxido compounds should happen to occur, rearrangement studies of the glycols so formed would be of interest, for in the case of the C_9–C_{11} oxide the only possible keto compound which could be produced would be the desired C_{11} ketone.

Therefore, it seemed pertinent to study methods for the direct conversion of these unsaturated cholenic acids to keto and glycol derivatives through the preparation and rearrangement of their oxides.
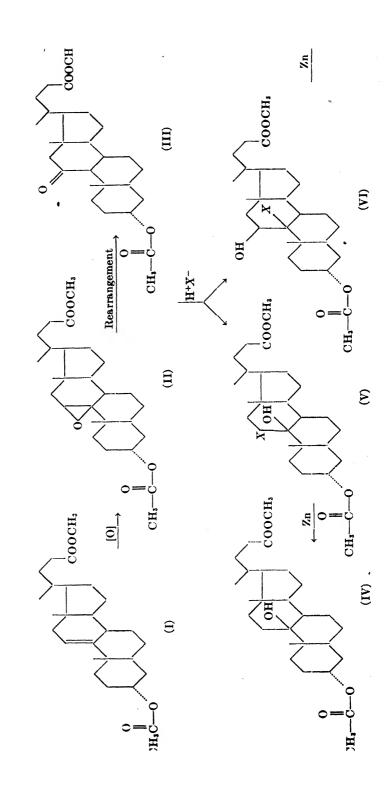
In order for us to obtain starting material for rearrangement studies, methods of preparing II and IX from the corresponding cholenic acids were investigated. The application of perbenzoic acid to the C_9–C_{11} double bond was shown by Chakravorty and Wallis[1] to yield an oxide mixed with a certain amount of starting material. Since in their hands difficulties were encountered in obtaining this oxide in a pure state, a method was sought whereby a stronger oxidizing agent could be used. The action of potassium permanganate, lead tetrapropionate, osmium tetroxide, hydrogen peroxide, and perbenzoic acid under a variety of conditions was investigated, and preliminary to our study of the action of certain of these reagents on II and IX, conditions for oxide formation
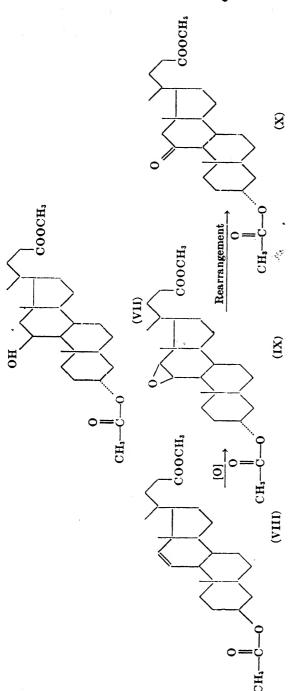
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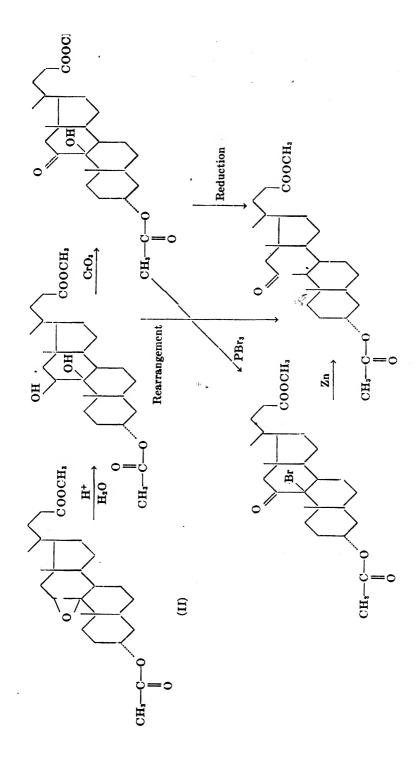
[1] Chakravorty, P. N., and Wallis, E. S., unpublished data.

COOCH$_3$

COOCH$_3$

OH

$\overset{O}{\underset{\parallel}{C}}$—CH$_3$

(VII)

COOCH$_3$

O

CH$_3$—C=O

(VIII)

[O]

COOCH$_3$

O

CH$_3$—C=O

(IX)

Rearrangement

CH$_3$—C=O

COOCH$_3$

O

(X)

648

COOC‖

OH

O

COOCH₃

CrO₂

CH₃—C═O

O

CH₃—C═O

O

Reduction

COOCH₃

O

CH₃—C═O

O

Rearrangement

PBr₃

COOCH₃

OH

OH

OH

CH₃—C═O

O

COOCH₃

H⁺
H₂O

O

CH₃—C═O

O

(II)

Zn

COOCH₃

O

Br

CH₃—C═O

O

were investigated with various derivatives of cholesterol, sitosterol, and ergosterol.

The use of lead tetrapropionate and of osmium tetroxide as the oxidizing agent led to results which are not of special interest in connection with this particular problem. However, the action of potassium permanganate and of perbenzoic acid did lead to results of sufficient interest to warrant a description of them at this time. With ergosteryl acetate, whose system of conjugated double bonds in Ring B had been protected by maleic anhydride,[2] hydrogen peroxide gave a pure C_{22}–C_{23}-oxido compound of melting point 224–228°. When the maleic anhydride addition product of dehydroergosteryl acetate was used as a starting material, a C_9–C_{11}, C_{22}–C_{23}-dioxido compound of melting point 256–257° was obtained in good yield when perbenzoic acid was the oxidizing agent.

Our next studies involved the use of potassium permanganate. The treatment at room temperature of cholesteryl benzoate with this reagent in acetic acid yielded a crystalline oxide which melted at 150° and was predominantly the β-oxide. A similar result was obtained with β-sitosteryl benzoate. In order to test this rapid method of oxide preparation on a bile acid derivative, the action of potassium permanganate on the known methyl $3(\alpha)$-acetoxy-$\Delta^{9,11}$-cholenate,[2] and on methyl $3(\alpha)$-acetoxy-Δ^{11}-cholenate, prepared by the method of Kendall *et al.* (5), was studied. With the latter compound a C_{11}–C_{12}-oxido derivative of melting point 142.5°, $[\alpha]_D^{20} = +56.9°$, was obtained in 65 per cent yield. These constants are in excellent agreement with those observed by Press and Reichstein (6) for the same compound prepared by the action of perbenzoic acid.

Extension of this method of permanganate oxidation to methyl $3(\alpha)$-acetoxy-$\Delta^{9,11}$-cholenate resulted in mixtures of two isomeric oxides. Chromatographic fractionation yielded a low melting oxide, m.p. 117–118°, $[\alpha]_D^{28} = +53.1°$, herein referred to as the β-oxide and identical with an oxide of melting point 117.5°, $[\alpha]_D^{20} = +50.1°$, prepared by the action of perbenzoic acid, and a high melting oxide of melting point 146–146.5°, $[\alpha]_D^{20} = +109°$, herein referred to as the α-oxide. Small amounts of starting material were also isolated. It was observed that the proportions of these two oxides varied with the conditions of the experiment. For example, higher yields of the α-oxide were obtained when the potassium permanganate was dissolved in 50 per cent aqueous acetic acid.

Chemical properties of cyclic oxides have been extensively studied and the opening of oxide rings has been effected with a variety of reagents.

[2] We would like to record at this point that in the preparation of ergosteryl acetate maleic anhydride prepared according to the method of Inhoffen (4) a new isomer of this compound was isolated. It has a melting point of 174–176°. $[\alpha]_D^{27} = -207°$ (22.2 mg. in 2 cc. of chloroform solution).

Space does not permit an exhaustive review of this work. Neither is it necessary to discuss in this paper the various mechanisms by means of which such oxides are rearranged to ketones or converted to glycols. It is pertinent, however, to cite a few examples from the field of the steroids which have had a direct bearing on this particular investigation. Thus, Fernholz (7) obtained 3(β)-acetoxycholestandiol-3,5 from cholesteryl acetate α-oxide when the latter compound was hydrogenated with the aid of a platinum catalyst. The same result was obtained by Chinaeva and Ushakov (8) when phenyl lithium was used as the reducing agent. When Grignard reagents are employed (8, 9), it has been observed that oxido cholesteryl acetate is converted to 6-ketocholestanol acetate. Rearrangement of C_5–C_6 oxides to C_6 ketones has also been effected by other methods (9, 10). It is interesting to note, however, that in many cases the two forms of the oxides in question behave differently (9, 10). Also, in some instances when acids such as hydrogen chloride are employed (9–11) chlorohydrins are isolated as the first products of the reaction.

Steroid oxides have also been hydrolyzed to glycols. Thus, Windaus and Westphalen (12) and Spring and Swain (9) have obtained C_5–C_6-glycols by the action of sulfuric acid upon cholesteryl acetate α-oxide and cholesteryl acetate β-oxide respectively. Similarly, acetic acid treatment of the α-oxide of 3(β)-acetoxyandrosten-5-one-17 has been shown by Ehrenstein (13) to yield 3,6-diacetoxy-5-hydroxyandrostanone-17.

With these experimental results in mind both hydrolytic and rearrangement studies of the oxides of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate and methyl 3(α)-acetoxy-Δ^{11}-cholenate were made. With the latter compound difficulty was encountered in rearrangement studies with such agents as magnesium bromide and zinc chloride. This fact rendered very doubtful the possibility of an attack on the C_9–C_{11} oxide by such reagents, since in an oxido compound of this kind the oxygen is protected by the C_{10}-methyl group, as is graphically demonstrated by the construction of a Fischer-Hirshfelder model. This idea was further substantiated by our initial experiments on catalytic hydrogenation. The C_9–C_{11} oxide is not susceptible to such reactions. Neither is the oxide ring sensitive to the action of common mineral acids. A chemical change was observed, however, when this oxide was treated with hydrogen fluoride.
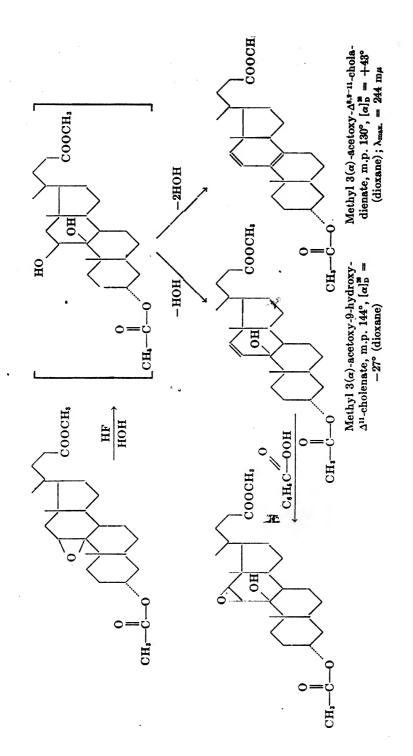
In order to determine conditions under which such a reagent was most effective in opening oxide rings we first studied its action on the α- and β-oxides of cholesteryl benzoate. It was soon observed that the major problems involved were concerned with temperature, solvent, and length of reaction time. Thus, it was found that at temperatures above $-50°$ almost complete decomposition of the oxide to non-crystalline material took place. Therefore, all reactions except one were carried out at tempera-

tures of $-80°$ over periods of time of less than 5 minutes. Platinum crucibles with covers were employed as reaction vessels and mixing was accomplished by addition of the crystalline oxide with stirring to the cold anhydrous liquid acid. An initial experiment with cholesteryl benzoate α-oxide in liquid hydrogen fluoride for 20 minutes showed that the reaction time was far too long. Only when the time of reaction was reduced to 5 minutes or less was it possible to obtain a crystalline product from this oxide. Under these conditions a crystalline compound was isolated which melted at $204°$, $[\alpha]_D^{20} = -12.7°$ in chloroform, and was shown by analysis to be a triol monobenzoate, differing, however, in configuration from the glycol obtained by sulfuric acid treatment of cholesteryl benzoate α-oxide.

In the hope that a ketonic product might be obtained by rearrangement various attempts were made to prevent hydrolysis of the oxide ring. Thus, instead of pouring the product of the reaction into water, studies were made of the quenching effect of ether, alcohol, and acetic anhydride, and of the solvent effect of carbon tetrachloride. Although in some instances the product of the reaction was not as clean cut, in all cases the same triol monobenzoate was produced, and when the reaction mixture was poured into absolute alcohol somewhat higher yields of the glycol derivative were obtained. It is of interest to note in this connection that the highest yields of the α-glycol monobenzoate were obtained when carbon tetrachloride was employed as a solvent. When acetic anhydride was used as solvent, no appreciable amount of reaction occurred.

Similar studies were carried out on cholesteryl benzoate β-oxide. Since Ehrenstein (13) has observed that the action of sulfuric acid on the two oxides of dehydroisoandrosterone acetate gives the same glycol, and, thus, has demonstrated that the hydrolysis proceeds by a different mechanism, it was hoped that there would be a possibility of obtaining a C_6 ketone by rearrangement of the β-oxide with hydrogen fluoride. However, repetition of the above reactions with the β-oxide again yielded a triol monobenzoate, but in this instance the mechanism of hydrolysis of the isomeric oxides proved to be the same and a different triol monobenzoate herein called the β-glycol monobenzoate of melting point $188°$, $[\alpha]_D^{20} = \pm 0°$ in chloroform, was obtained. Since this glycol monobenzoate is also different from the one obtained by Spring and Swain (9) by the action of sulfuric acid on the α-oxide of cholesteryl benzoate, we can conclude that three of the four possible isomeric glycols have been accounted for.

Our next experiments were carried out on the "β-oxide" of methyl $3(\alpha)$-acetoxy-Δ^{11}-cholenate (5). Conditions for the reaction were the same as those previously employed except that the reaction time was confined to 1 minute. On working up the product, methyl $3(\alpha)$-acetoxy-11,12-dihydroxycholanate was obtained in yields of 35 per cent. The

HF / HOH

−HOH

−2HOH

C₆H₅C—OOH

Methyl 3(α)-acetoxy-9-hydroxy-Δ¹¹-cholenate, m.p. 144°, [α]²⁵_D = −27° (dioxane)

Methyl 3(α)-acetoxy-Δ⁸,⁹⁻¹¹-choladienate, m.p. 130°, [α]²⁵_D = +43° (dioxane); λ_max = 244 mμ

pure compound melted at 146°, $[\alpha]_D^{20} = +59.4°$ in absolute alcohol. Saponification of this material followed by methylation yielded a compound of melting point 135°, $[\alpha]_D^{19} = +47.5°$ in alcohol.

The action of hydrogen fluoride on the "β-oxide" of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate was found to proceed in a somewhat different manner. Preliminary runs with the "β-oxide" when the reaction time was 5 minutes led to the formation of non-crystallizable oils. When, however, the time was decreased to 1 minute or less, the oil so obtained yielded, upon chromatographic fractionation, two crystalline products of melting point 130°, $[\alpha]_D^{20} = +43°$ in dioxane, and 144°, $[\alpha]_D^{26} = -27°$ in dioxane, respectively. The lower melting compound was shown by analysis to contain no oxygen in Ring C. Absorption spectra showed the presence of a conjugated system of double bonds. The higher melting product was shown by analysis to contain an oxygen atom in Ring C. It was found to be stable to chromic acid, but perbenzoic acid treatment showed the presence of a double bond. Thus it is our belief that the reaction of hydrogen fluoride on the β-oxide of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate proceeds as in the accompanying structures.

C_{22}–C_{23} Oxide of Ergosteryl Acetate Maleic Anhydride—1.8 gm. of ergosteryl acetate maleic anhydride (m.p. 216°), prepared according to the method of Inhoffen (4) with slight modifications, were dissolved in 6 cc. of acetic acid and treated for 5 hours on the water bath with 1 cc. of 30 per cent hydrogen peroxide. On working up the product crystals were obtained which melted unsharply at 221–224°. On recrystallization three times from acetone and after careful drying the pure compound melted at 224–228°; $[\alpha]_D^{22} = -13°$ (20 mg. in 2 cc. of dry chloroform solution).

Analysis—$C_{34}H_{48}O_6$. Calculated, C 73.81, H 8.72; found, C 73.88, H 8.78

New Isomeric Form of Ergosteryl Acetate Maleic Anhydride—The mother liquors from the Inhoffen (4) preparation of ergosteryl acetate maleic anhydride were concentrated and then taken up in methyl alcohol. Crystals were obtained which on further recrystallization to constant melting point from methanol melted at 174–176°; $[\alpha]_D^{27} = -207°$ (20 mg. in 2 cc. of dry chloroform solution).

Analysis—$C_{34}H_{48}O_5$. Calculated, C 76.08, H 9.02; found, C 76.18, H 9.14

C_9–C_{11}, C_{22}–C_{23} Dioxide of Dehydroergosteryl Acetate Maleic Anhydride—Dehydroergosteryl acetate maleic anhydride was prepared by the method of Murke (14, 15). 0.4 gm. of this substance was treated for 3 days with a 2-fold excess of perbenzoic acid dissolved in chloroform. On working

up the product 0.1 gm. of material of melting point 218–235° was obtained. Five recrystallizations from ethyl acetate gave crystals which melted at 256–258°; $[\alpha]_D^{24} = -8°$ (20 mg. in 2 cc. of dry chloroform solution). It can be crystallized also from acetic acid.

Analysis—$C_{34}H_{46}O_7$. Calculated, C 71.80, H 8.51; found, C 71.77, H 8.37

Cholesteryl Benzoate β-Oxide—A solution of 1.3 gm. of potassium permanganate (1.1 M) in 12 cc. of water and 20 cc. of acetic acid was added at room temperature to 5.0 gm. of cholesteryl benzoate dissolved in 100 cc. of glacial acetic acid and 20 cc. of chloroform. After 20 minutes the reaction mixture was worked up. Crystallization of the crude material from ethyl acetate gave 1.7 gm. of needles of melting point 147–150°. Additional amounts of material were obtained by concentration of the mother liquors. A mixed melting point determination with a sample of the β-oxide of melting point 149–150° prepared by the usual method gave no depression.

β-Sitosteryl Benzoate β-Oxide—A solution of 5.0 gm. of β-sitosteryl benzoate (m.p. 145–147°) in 100 cc. of acetic acid and 50 cc. of chloroform was treated at room temperature for 15 minutes with 1.2 gm. of potassium permanganate dissolved in 90 cc. of acetic acid and 10 cc. of water. Bisulfite decolorization was followed by addition of water, chloroform extraction, washing, drying, and removal of solvent. The crystalline product so obtained melted at 181–184°; $[\alpha]_D^{20} = +8.6°$ (39.2 mg. in 5 cc. of chloroform solution).

Methyl 3(α)-Acetoxy-11(β),12(β)-oxidocholanate—3 gm. of methyl 3(α)-acetoxy-Δ^{11}-cholenate were dissolved in 30 cc. of glacial acetic acid and treated in one portion with 2.25 gm. of potassium permanganate in 170 cc. of 90 per cent acetic acid. After 2 minutes the reaction mixture was worked up in the usual manner. Crystallization from methanol gave 1.9 gm. of a product which melted at 134–137°. Further recrystallization from methanol gave a product of constant melting point of 140–142°; $[\alpha]_D^{20} = +56.9°$ (10.2 mg. in 2 cc. of absolute alcohol).

Analysis—$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 72.78, H 9.38

Methyl 3(α)-Acetoxy-9(β),11(β)-oxidocholanate—Methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate, m.p. 133–135°, $[\alpha]_D^{25} = +62.9°$, was prepared from 3-hydroxy-$\Delta^{9,11}$-cholenic acid (1, 2). A solution of 280 mg. of this ester in 0.5 cc. of chloroform was treated for 6 hours at room temperature with 1.2 cc. (1.2 N) of perbenzoic acid in chloroform. On working up the product crystals were obtained which after two recrystallizations from dilute acetone gave 200 mg. of plates melting at 115.5–117.5°; $[\alpha]_D^{28} = +50.1°$ (16.8 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 72.55, H 9.64

A 350 mg. sample of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate (m.p. 133–137°; $[\alpha]_D^{28} = +69.4°$) prepared by a method developed by Schwenk (private communication) was subjected to the same procedure. The yield of crystalline product was 225 mg., m.p. 116.5–118.5°; $[\alpha]_D^{28} = +53.1°$ (17.6 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 72.76, H 9.70

Several experiments were carried out with potassium permanganate as the oxidizing agent. In all cases a mixture of products was obtained which consisted of starting material and two isomeric oxides in varying amounts, depending on the conditions of the experiment, which could be separated by chromatographic fractionation.

In one experiment 600 mg. of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate were dissolved in 12 cc. of glacial acetic acid and treated in one portion with 0.15 gm. of potassium permanganate in 12 cc. of 90 per cent acetic acid. After 20 minutes the reaction mixture was worked up in the usual manner. Crystals were obtained which melted unsharply at 123° and were obviously a mixture. Yield, 0.310 gm.; $[\alpha]_D^{18} = +64.0°$ (17.5 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 74.15, H 9.95

In another experiment 1.3 gm. of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate were dissolved in 50 cc. of glacial acetic acid and treated at room temperature with 0.40 gm. of potassium permanganate dissolved in 4 cc. of water and 36 cc. of glacial acetic acid. After 20 minutes bisulfite was added and the product worked up. Crystallization from ether gave 0.54 gm. of a material melting at 117–120°. A second crop of crystals (0.53 gm.) was obtained on concentration of the mother liquors. This material melted at 112–118°. Evaporation of the mother liquors gave 0.5 gm. of an oily residue.

These three fractions were then dissolved in benzene and separately submitted to chromatographic analysis. Specially treated Merck's alumina was used as the adsorbent and the eluent was a mixture of benzene (96 per cent) and ether (4 per cent) under a pressure head of 220 mm. of mercury. On developing the chromatogram Fraction I yielded 415 mg. of a crystalline product melting at 117–123° and 64 mg. of a compound melting unsharply at 141°. Several recrystallizations of the low melting fraction from methanol gave crystals which melted at 133–135° (identical with the starting material). Purification of the higher melting fraction led to a compound melting at 146° (see below). From Fraction II were obtained 169 mg. of a material melting at 106–112°, which on recrystallization gave crystals of the β-oxide of melting point 116–118°. A small

amount of starting material (37 mg.) was also isolated from this fraction. On similar treatment Fraction III yielded 165 mg. of crude β-oxide (m.p. 106–110°), which on purification melted at 117°, and approximately 200 mg. of an unidentified oil.

Methyl 3(α)-Acetoxy-9(α),11(α)-oxidocholanate—In the experiment described above only small amounts of this isomeric oxide were obtained. We have observed that the isolation of this isomer from the reaction mixture is easier if the oxidation is carried out in the presence of more water. Thus, the addition at room temperature of a slight excess of a 5 per cent solution of potassium permanganate, dissolved in 50 per cent aqueous acetic acid, to 500 mg. of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate gave a product which upon chromatographic analysis, as described above, and recrystallization from dilute acetone melted at 146–146.5°; $[\alpha]_D^{20} = +109.1°$ (16.0 mg. in 2 cc. of absolute alcohol solution); yield, 85 mg.

Treatment of 10 mg. of this product with chromic acid (3 mg. in 1 cc. of 95 per cent acetic acid) for 12 hours gave back starting material which melted at 146°. Similar treatment of 39 mg. of the same material in chloroform with perbenzoic acid for 20 hours showed that it was also inert to the action of this reagent. Titration showed that no perbenzoic acid had been consumed and, on working up the product, 25 mg. of crystals were recovered which melted at 145° and were found to be identical with the starting material.

A mixed melting point determination of a sample of this compound, m.p. 145°, $[\alpha]_D^{20} = +109°$, with methyl 3(α)-acetoxy-12-ketocholanate, m.p. 148–150°, $[\alpha]_D^{27} = +107.5°$, was 136–142.°

Analysis—$C_{27}H_{42}O_5$. Calculated, C 72.61, H 9.48; found, C 72.37, H 9.90

Action of Hydrogen Fluoride on Cholesteryl Benzoate α-Oxide—2 gm. of cholesteryl benzoate α-oxide of melting point 163–164°, prepared by the action of perbenzoic acid on cholesteryl benzoate, were added with immediate solution to 20 cc. of liquid hydrogen fluoride in a platinum crucible at −50°. An orange color developed and after 20 minutes the solution was poured into 600 cc. of ice water. On working up the product, no crystalline material could be isolated.

2 gm. of cholesteryl benzoate α-oxide were treated as described above except that the reaction time was only 5 minutes and the temperature was −80°. Crystallization of the product from acetone gave plates which melted at 199–204°; yield, 0.4 gm. Two recrystallizations gave plates melting at 204–205°; $[\alpha]_D^{20} = -12.7°$ (18.8 mg. in 2 cc. of chloroform solution).

Analysis—$C_{34}H_{52}O_4$. Calculated, C 77.81, H 9.99; found, C 77.44, H 10.07

1 gm. of cholesteryl benzoate α-oxide was added to a solution of 5 cc. of liquid hydrogen fluoride in 25 cc. of carbon tetrachloride at $-20°$. After 5 minutes the product was worked up in the usual manner. Crystals were obtained which melted at 202–204°; yield, 0.3 gm.

1 gm. of cholesteryl benzoate α-oxide was added to a solution of 3 cc. of liquid hydrogen fluoride in 25 cc. of acetic anhydride at $-73°$. Since there was no apparent change, the temperature was raised to $+45°$, and then the solution was poured into water. On working up the product, 0.75 gm. of starting material was isolated.

1 gm. of cholesteryl benzoate α-oxide was added to 7 cc. of liquid hydrogen fluoride at $-80°$. After 5 minutes the solution was poured into 50 cc. of acetic anhydride at $-50°$. Crystals were obtained which melted at 194–198°. Recrystallization from acetone and ethyl acetate gave plates which melted at 204–205°.

0.5 gm. of cholesteryl benzoate α-oxide was added to 15 cc. of liquid hydrogen fluoride at $-80°$. After 3 minutes the solution was poured into a large volume of ether at $-80°$. A vigorous reaction took place, after which the solution was worked up. An oil was obtained which crystallized with difficulty. Four recrystallizations from acetone gave a crystalline material which melted at 190–195° and though not pure contained for the most part only the triol monobenzoate.

Action of Hydrogen Fluoride on Cholesteryl Benzoate β-Oxide—A portion (0.9 gm.) of cholesteryl benzoate β-oxide (m.p. 149–150°) was added to 12 cc. of liquid hydrogen fluoride at $-80°$. After 5 minutes the solution was poured into water and worked up in the usual manner. Needles were obtained from acetone which after three recrystallizations melted at 186–188°; $[\alpha]_D^{19} = \pm0.0°$ (17.8 mg. in 2 cc. of chloroform).

Analysis—$C_{34}H_{52}O_4$. Calculated, C 77.81, H 9.99; found, C 77.51, H 10.03

Action of Hydrogen Fluoride on Methyl 3(α)-Acetoxy-11(β),12(β)-oxido-cholanate. Preparation of Methyl 3(α)-Acetoxy-11,12-dihydroxycholanate—To 7 cc. of liquid hydrogen fluoride at $-80°$ was added 0.3 gm. of methyl 3(α)-acetoxy-11(β),12(β)-oxidocholanate. After 5 minutes the reaction mixture was poured into water and worked up in the usual manner. The crude crystalline product weighed 0.10 gm. and melted at 133–136°. Three crystallizations gave a compound of constant melting point, 146–147°; $[\alpha]_D^{20} = +59.4°$ (17.5 mg. in 2 cc. of absolute alcohol solution).

Analysis—$C_{27}H_{44}O_6$. Calculated, C 69.79, H 9.56; found, C 69.50, H 9.46

When a mixed melting point determination was made of this compound with the starting material, a depression was observed. Saponification of 0.2 gm. of this product with 10 cc. of 2.5 per cent methanolic potassium

hydroxide gave a non-crystalline oil. Treatment of this oil, however, with diazomethane gave a crude crystalline product of melting point 120–129°. Five crystallizations from acetone and ether gave a compound melting at 134.5–135°; $[\alpha]_D^{19} = +47.5°$ (16 mg. in 2 cc. of absolute alcohol solution).

Action of Hydrogen Fluoride on Methyl 3(α)-Acetoxy-9(β),11(β)-oxidocholanate—(a) To 7 cc. of liquid hydrogen fluoride cooled to −80° were added 190 mg. of methyl 3(α)-acetoxy-9(β),11(β)-cholanate, m.p. 115.5–117.5°. After 30 seconds the solution was poured into water and worked up as usual. A non-crystalline oil was obtained. Yield, 148 mg. This material was dissolved in 10 cc. of benzene and adsorbed on a column of 6 gm. of specially prepared Merck's alumina. The chromatogram was developed under a pressure of 220 mm. of mercury, first by a mixed solvent of 70 and 30 per cent pentane followed by pure benzene, and finally by a mixed solution of benzene and diethyl ether in varying proportions. Two distinct fractions were obtained. Fraction I when crystallized from dilute acetone melted at 129–130°. Fraction II when crystallized from dilute acetone melted at 143–144°.

(b) The above experiment was repeated with 200 mg. of methyl 3(α)-acetoxy-9(β),11(β)-oxidocholanate of melting point 116.5–118°. Solution was effected in 4 cc. of liquid hydrogen fluoride at −80° and after 30 seconds the product was worked up in the usual manner. A non-crystalline product (165 mg.) was obtained which on chromatographic fractionation again gave two compounds of melting point 129–129.5° and 143–143.5° respectively.

(c) The mother liquors from the crystallizations in experiments (a) and (b) were united, evaporated to dryness, and again submitted to chromatographic analysis. The non-crystalline material was dissolved in 15 cc. of benzene and 10 cc. of pentane and was placed on the column in the manner described above.

On development of the chromatogram further amounts of Fractions I and II were obtained. The total yield of Fraction I obtained in the two experiments was 94 mg. A sample of the product which was submitted for analysis melted at 130–131°; $[\alpha]_D^{26} = +43.0°$ (6.1 mg. in 0.50 cc. of dioxane solution).

Analysis—$C_{27}H_{40}O_4$ (a diene). Calculated, C 75.66, H 9.41; found, C 75.97, H 9.6

The absorption spectrum showed a maximum at wave-length 244 mμ, with molecular extinction coefficient of $\epsilon = 420$. The low value for ϵ suggests that the double bond originally found in the 8,9 position had largely migrated to the 14,15 position.

The total yield of Fraction II obtained in the two experiments was

227 mg. A sample of the pure product which was submitted for analysis melted at 143–144°; $[\alpha]_D^{26} = -27.0°$ (5.9 mg. in 0.50 cc. of dioxane solution).

Analysis—$C_{27}H_{42}O_5$ (an unsaturated diol monoacetate methyl ester)
Calculated, C 72.61, H 9.48; found, C 72.41, H 9.56

Action of Chromic Acid on Methyl 3(α)-Acetoxy-9-hydroxy-Δ^{11}-cholenate— 10 mg. of methyl 3(α)-acetoxy-9-hydroxy-Δ^{11}-cholenate were dissolved in 0.5 cc. of acetic acid and treated with 2.5 mg. of chromic acid dissolved in 1 cc. of 95 per cent acetic acid. The solution was allowed to stand for 12 hours, and then worked up in the usual manner. The non-crystalline residue so obtained was dissolved in hot acetone from which, on standing, crystals of starting material were obtained of melting point 143–144°. It can, thus, be concluded that the nature of oxygen in Ring C is not that of a secondary hydroxyl group.

Action of Perbenzoic Acid on Methyl 3(α)-Acetoxy-9-hydroxy-Δ^{11}-cholenate —8 mg. of the above material were dissolved in 2 cc. of chloroform solution of perbenzoic acid (1 N). After 6 hours standing at room temperature the reaction mixture was tested with potassium iodide solution. Titration showed no appreciable perbenzoic acid to be present. Thus the presence of a double bond in Ring C is indicated.

SUMMARY

Certain steroid oxides have been prepared and improved conditions for oxide formation have been investigated.

The chemical properties of these cyclic oxides have been studied and the opening of the oxide ring with hydrogen fluoride has been effected. Both hydrolytic and rearrangement studies of the oxides of methyl 3(α)-acetoxy-$\Delta^{9,11}$-cholenate and methyl 3(α)-acetoxy-Δ^{11}-cholenate have been carried out and the nature of the products formed has been determined.

A new isomeric form of ergosteryl acetate maleic anhydride has been prepared and the dioxide of dehydroergosteryl acetate maleic anhydride has been characterized.

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EXPERIMENTAL STUDIES IN THE STEROIDS. THE OXIDATION OF METHYL 3(α)-ACETOXY-11-KETO-12-BROMOCHOLANATE*

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An important intermediate in the synthesis of Compound E (I) is a compound whose structure is that indicated in formula II. This latter compound (m.p. 162–163°) has already been prepared by Sarett (1) by step by step degradation methods. In this paper we wish to describe certain oxidation experiments by means of which we have been able to remove the cholanic acid side chain in one operation. For this purpose[1] methyl 3(α)-acetoxy-11-keto-12-bromocholanate was prepared by the method of Reich and Reichstein (2) as modified by Sarett (1).

In our hands this compound melted at 176–178°. When it was submitted to oxidation by a method similar to that of Ruzicka and Goldberg (3) and the products of the oxidation worked up in such a manner as to remove the neutral ketonic material free from bromine, there was isolated in small yields by chromatographic methods Compound II, m.p. 158–160°.

EXPERIMENTAL

10 gm. of methyl 3(α)-acetoxy-11-keto-12-bromocholanate, m.p. 176–178°, were dissolved in 355 cc. of glacial acetic acid and to the solution, which was kept at 85–90°, there was added dropwise over a period of 4 hours, with stirring, a solution of 14.4 gm. of chromic acid dissolved in 14.4 cc. of water and 46.6 cc. of glacial acetic acid. After the addition was completed, the mixture was stirred for another 4 hours at a temperature of 85–90°. To the solution 45 cc. of methyl alcohol were then added and the contents were evaporated to a small volume (75 to 100 cc.) under diminished pressure. Water was then added and the material was ex-

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[1] This particular compound was chosen because it was our belief that the introduction of bromine to the 12 position α to the ketone group would sufficiently stabilize Ring C so that during the course of oxidation the ring would not be ruptured.

tracted with ether. The ether layer was washed with a 10 per cent solution of potassium carbonate several times to remove acidic materials. It was then dried and the ether evaporated completely on a steam bath. A white crystalline residue was obtained (weight, 5.2 gm.).

This residue was taken up in 200 cc. of acetic acid and debrominated in the usual manner with zinc dust. After debromination was complete, the material was poured into water and extracted with ether. On working up the ether-soluble product, a pale yellow viscous oil (3.78 gm.) was obtained.

Removal of non-ketonic neutral material was effected in the following manner. The pale yellow oil described above was taken up in 200 cc. of benzene and the solution evaporated to dryness. Methyl alcohol (14 cc.) was added and a white crystalline material separated. This was dissolved

(I)   (II)

under a reflux and to the solution were added 1.75 gm. of Girard's Reagent T and 1.4 cc. of glacial acetic acid. The refluxing was continued for 1 hour. After cooling, 72 cc. of ice water were added, and, with shaking in an ice bath, 140 cc. of 0.135 N NaOH (ice-cold) were added in small portions. The contents (still acidic) were shaken for an additional 10 minutes and then extracted with ether. The ethereal solution was washed and worked up in the usual manner. Weight of the non-ketonic neutral fraction, 3.51 gm.

The aqueous solution from the above experiment was treated with 3.67 cc. of 6 N H_2SO_4, calculated to bring the pH to 2.9 to 3.0. After about 45 seconds it was extracted with ether and the ether layer worked up in the usual manner. A crude crystalline product (50 mg.), of melting point 125–128°, was obtained (melting point of methyl 3(α)-acetoxy-11-keto-cholanate 130–132°).

To the aqueous portion after the removal of the product described above there was added enough ice-cold sulfuric acid to bring the acidity of the solution to approximately 0.75 N in H_2SO_4. After standing overnight at room temperature it was extracted with ether and the ether-soluble fraction worked up. To the oily residue so obtained there were added 75 cc. of water and the contents of the flask were submitted to steam distillation in order to remove volatile ketonic material. The non-volatile residue (197 mg.) was taken up in 10 cc. of benzene, and the solution evaporated in order to free the material from traces of water. It was then reacetylated by treatment with 1.8 cc. of acetic anhydride and 2.0 cc. of pyridine at the temperature of the water bath. The reacetylated product was submitted to chromatographic analysis, and the chromatogram developed with a mixture of petroleum ether and diethyl ether. Two fractions were obtained. Fraction I was rechromatographed and then taken up in a small volume of ethyl acetate. Pentane was added and the solution was seeded with an authentic specimen of the 3(α)-acetoxy-11,17-diketone prepared by Sarett (1). Crystalline rosettes soon formed and after several days standing were filtered and recrystallized. The product so obtained (10 mg.) melted at 158–160° and with an authentic specimen furnished us by Dr. Sarett gave no depression of melting point.

SUMMARY

The oxidation of methyl 3(α)-acetoxy-11-keto-12-bromocholanate with chromic acid has been studied. Small yields of 3(α)-acetoxycholandione-11,17 have been isolated, m.p. 158–160°, identical with the product of melting point 159–160° prepared by Sarett by step by step degradation methods.

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EXPERIMENTAL INVESTIGATIONS IN THE CHEMISTRY OF RING C IN DESOXYCHOLIC ACID*

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In 1938 it was reported by Marker and Lawson (1) that when $3(\alpha)$-acetoxy-12-ketocholanic acid is brominated at 70° and the resulting crude bromoketone refluxed with methanolic potassium hydroxide a compound melting at 196° is obtained in about 35 per cent yield. To this substance was assigned the structure $3(\alpha)$,11-dihydroxy-12-ketocholanic acid (I). These authors also described the preparation of a "3-monoacetate" (II), of melting point 268°, in unspecified yield. By certain modifications of their method the same dihydroxyketocholanic acid has been prepared by Longwell and Wintersteiner (2). However, these latter workers have advanced evidence that the "3-monoacetate" of Marker and Lawson actually is not II but is either a 3-acetoxy lactone or, more probably, a bimolecular anhydride diacetate. Longwell and Wintersteiner obtained the true monoacetate on refluxing with 33 per cent acetic acid. Their product melted at 106°, and the analysis agreed with a hemihydrate of a dihydroxyketocholanic acid monoacetate.

At the time the present investigation was begun, no method for the introduction of oxygen, either as a secondary hydroxyl group or as a ketone, into the C_{11} position of the steroid nucleus was known. The failure of various orthodox methods led us to an investigation of the possibility of preparing an 11-ketosteroid, namely $3(\alpha)$-hydroxy-11-ketocholanic acid (VII), by first opening Ring C to a $C_{11}\|C_{12}$-tricarboxylic acid (III), transforming the carboxyl attached to C_{13} to a $—CH_2 \cdot COOH$ group, and then closing Ring C with eventual formation of the desired 11-ketone.

The proposed series of reactions, as originally envisioned by us, is represented in structures I to VII. The starting material (I) was prepared according to Longwell and Wintersteiner (2). No particular difficulty was anticipated in opening Ring C by oxidation with chromic acid, since Reichstein (3) had converted in this manner methyl 11-hydroxy-12-ketocholanate to the corresponding dibasic acid in excellent yield. Compound III pos-

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(I)   (II)

(IV)   (III)

(V)   (VI)

sesses carboxyl groups attached to secondary, tertiary, and quaternary carbon atoms and it was hoped that the rates of esterification of these three carboxyls might be sufficiently different to effect the partial esterification leading to the monobasic acid (IV). For molecules of such complexity and sensitivity the only method of lengthening the carbon chain of the acid (*i.e.* conversion of IV to V) seriously to be considered is that involving the Wolff rearrangement. Once this has been accomplished, it would appear that the product could be converted to 3(α)-hydroxy-11-keto-cholanic acid (VII) either by a Dieckmann condensation via the intermediate (VI) or by pyrolytic ring closure of the dibasic acid from V.

In our hands, however, the low yield (less than 20 per cent) obtained in the preparation of the necessary monoacetate of the dihydroxyketocholanic acid compelled us to turn our attention to other methods for the oxidative cleavage of the grouping in Ring C which would not affect the unprotected C_3-hydroxyl group. It is interesting to note in this connection that recently

Longwell and Wintersteiner (2) in their studies on the preparation of II also obtained this compound in low yields.

The Criegee oxidation of glycols (4) with lead tetraacetate in glacial acetic acid has recently been extended by Baer (5) to the cleavage of α-hydroxy ketones, the only significant change being that 90 per cent acetic acid is used as solvent. However, when applied to the methyl ester of the dihydroxyketocholanic acid (VIII), this technique gave us in only poor yield 3(α)-hydroxy-11∥12-cholane aldehyde 11-diacid-12,24 (IX), of melting point 246–253°.

In an effort to obtain IX in better yield, it was decided to study the periodic acid oxidation of 3(α),11-dihydroxy-12-ketocholanic acid. The reaction was carried out in aqueous solution buffered with sodium bicarbonate and containing a few per cent of ethyl alcohol. Because of its solubility the sodium salt was used. The oxidation, which was followed by titrating the unused periodate with arsenite, proceeded essentially to com

pletion when the solution stood overnight at room temperature. In our hands, the yield of the hydroxy aldehyde dibasic acid (IX) was 70 per cent. The analytically pure product melted at 248–254°.

Acetylation and chromic acid oxidation of IX gave in 42 per cent yield $3(\alpha)$-acetoxy-11∥12-cholane triacid-11,12,24 (III), m.p. 229–232°. The aldehyde group, however, proved quite resistant to oxidation, 4 days at 45° being required to convert it into a carboxyl group. Saponification of this acetoxy tribasic acid gave a hydroxy tribasic acid of melting point 249–253°.

As has been previously indicated, it was expected that the carboxyl group on C_{13} in the tribasic acid, being attached to a quaternary carbon atom, would not be esterified in the acid-catalyzed reaction with methanol, whereas the carboxyl group attached to C_9 might be esterified by this treatment. On reacetylation this would lead to the acid diester (IV).

(X)   (XI)

Actually, we found that neither of the carboxyl groups of Ring C was esterifiable with methanol and acid. Only the monoester, $3(\alpha)$-acetoxy-11∥12-cholane diacid 11,12-methyl ester-24 (X), m.p. 208–209°, was obtained. On refluxing with thionyl chloride, this latter compound gave an anhydride (XI), m.p. 206°.

Our inability to prepare the desired monoacid diester (IV) led us to a study of the aldehydic compound (IX). It has already been pointed out that the aldehyde group in this molecule is relatively unreactive. However, it seemed advisable to determine its behavior toward certain other reagents, as for example alkali, methyl alcohol and mineral acids, acetic anhydride, and diazomethane.

It was found to be completely unreactive toward all these reagents. Thus, when treated with an excess of diazomethane, the hydroxy aldehyde diacid gave in excellent yield $3(\alpha)$-hydroxy-11∥12-cholane aldehyde 11-dimethyl ester-12,24 (XII), m.p. 128.5–129.5°. This compound was easily

acetylated to the acetoxy aldehyde dimethyl ester (XIII), m.p. 127–128°. On standing overnight in methanol containing 2 per cent of concentrated sulfuric acid, the hydroxy aldehyde diacid was converted to 3(α)-hydroxy-11‖12-cholane aldehyde 11-acid 12-methyl ester-24 (XIV), m.p. 148–149.5°;

under these conditions no dimethyl ester was formed.   Acetylation of XIV gave 3(α)-acetoxy-11‖12-cholane aldehyde 11-acid 12-methyl ester-24 (XV), m.p. 168–171°.

It was also observed that vigorous treatment with alkali failed to saponify the quaternary carbomethoxy group attached at C_{12}.   Thus the reaction

of the dimethyl ester (XII) with either potassium carbonate in dilute methanol at room temperature or with boiling methanolic potassium hydroxide gave only 3(α)-hydroxy-11‖12-cholane aldehyde 11-methyl ester 12-acid-24 (XVI), of melting point 168–170°.

The C_{11}-aldehyde, however, did react in typical fashion with hydroxylamine to form an oxime, 3(α)-acetoxy-11‖12-cholane aldoxine 11-dimethyl ester-12,24 (XVII), m.p. 148.5–149.5°.

The reactions outlined above indicate that the acetoxy aldehyde acid ester (XV) would be a suitable starting point for a Wolff rearrangement leading to the desired homoacid or ester (XX) as indicated below, provided that the diazoketone (XIX) could be prepared. Compound XX could then be converted by chromic or peracetic acid oxidation of the aldehyde group to V or one of its suitable derivatives which would be amenable to ring closure, as previously indicated.

(XVII)

For this purpose Compound XV was converted to the corresponding acid chloride (XVIII) by treatment with thionyl chloride. The reaction was found to be almost quantitative and the chloride so produced was easily obtained in crystalline form of melting point 137–139°. When refluxed with methanol in the presence of a little mineral acid, it was converted to XII, the C_{12}-carboxyl group being esterified by this sequence of reactions. When a solution of the acid chloride, however, was added to cold ether containing an excess of diazomethane, no reaction took place. Nor could the formation of the diazoketone be effected by changing solvents or working at higher temperatures. Apparently the reaction is completely prevented by the extreme steric hindrance of the quaternary acid chloride. This observation is in agreement with the findings of Litvan and Robinson (6) who could not form a diazoketone from the sterically hindered camphoric acid α-methyl ester β-acid chloride.

In an effort to find a method of preparation of sterically hindered diazo-

ketones of this type the above reaction was studied in pyridine. Since
the formation of a diazoketone may well involve the attack of a positive
RCO^+ ion on the diazomethane molecule, followed by loss of a proton,
and since pyridine forms with many acid chlorides reactive salts of the
type $(RCOC_5H_5N)^+Cl^-$, we undertook to prepare such a complex between
the organic base and the steroid acid chloride, hoping that this complex
might be isolated and then caused to react rapidly with an excess of diazo-
methane, forming the diazoketone.

When the above acid chloride was heated for 5 days at 45° in carefully
dried pyridine, removal of the solvent gave an oily yellow residue which

(XVIII)   (XIX)

(XX)

crystallized on addition of toluene. A small portion of this material,
washed with ether by centrifuging and dried in a stream of air, melted
unsharply at about 195° and gave a strongly positive Beilstein test. It was
at first believed that this was the desired salt, $(RCOC_5H_5N)^+Cl^-$. Further
study showed, however, that it was an equimolecular mixture of pyridine
hydrochloride and a steroid derivative, designated as Compound A.

This substance is neutral and is a fine white powder, melting at 199–201°.
It contains neither nitrogen nor halogen. On the basis of its mode of
formation, analysis, and molecular weight, together with certain other
chemical properties described below, we believe this compound to have the
structure XXI.

The analytical data eliminate any monomeric structures; that the molecule is a dimer is shown by its observed molecular weight of 948, as compared with the calculated value of 952. When Compound A was refluxed with absolute methanol containing 2 per cent of concentrated sulfuric acid, neutral and acid fractions of almost exactly equal molecular amounts were obtained. The neutral fraction was identified as XII. This result is consistent only with an acid anhydride structure. Final confirmation of structure XXI was obtained by treating Compound A with hydroxylamine. The product, which melted at 181.5–183°, was found to be a dioxime.

(XXI)

EXPERIMENTAL[1]

3(α)-Hydroxy-11‖12-cholane Aldehyde 11-Diacid-12,24 (IX)—Exactly 5.00 gm. of 3(α),11-dihydroxy-12-ketocholanic acid were dissolved in 35 cc. of hot ethyl alcohol and 51 cc. (10 per cent excess) of 0.24 N sodium hydroxide were added. The solution was transferred to a 500 cc. volumetric flask and 4.6 gm. of sodium bicarbonate were added and dissolved.

To a solution of 3.09 gm. (10 per cent excess) of periodic acid, H_5IO_6, in 125 cc. of water, was added slowly and with swirling a solution of 4.6 gm. of sodium bicarbonate in about 75 cc. of water. The resulting mixture was added slowly and with constant swirling to the material in the volumetric

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[1] All melting points recorded in this paper are uncorrected. All rotations were taken in acetone, with a 1 dm. semimicro tube; c = 0.8 to 1.3.

flask, and the whole was made up to 500 cc. with water. The solution was clear at this point.

The course of the reaction was followed by withdrawing 1 cc. aliquot portions, adding a little sodium bicarbonate and a few crystals of potassium iodide, and titrating the liberated iodine with 0.01 N arsenite solution. After standing overnight at room temperature the reaction mixture was warmed to about 45° and maintained at that temperature until 97 per cent of the theoretical amount of H_5IO_6 had reacted.

The solution was diluted with 2 or 3 volumes of water, acidified to Congo red with hydrochloric acid, and extracted with about 1 liter of ether. The ethereal solution was washed with water until neutral, during which operation a small amount of material crystallized out and was collected separately. The ether solution was dried with sodium sulfate for 30 minutes, filtered, and concentrated to about 100 cc.; at this point most of the product had separated in crystalline form. The product was filtered and washed with dry ether. The yield of the first crop was 2.55 gm. of white crystals, m.p. 240–245°. Two additional crops were obtained by further concentration of the mother liquor. The total yield of product melting above 240° was 3.63 gm. (70 per cent). A sample for analysis was crystallized from dilute ethyl alcohol and from acetone until it melted constantly at 248–254°. $[\alpha]_D^{17} = +67.2°$.

Analysis—$C_{24}H_{38}O_6$. Calculated, C 68.21, H 9.06; found, C 68.39, H 9.28

Methyl 3(α),11-dihydroxy-12-ketocholanate (0.25 gm.) was prepared by treatment of the corresponding acid with diazomethane. It was dissolved in 8 cc. of 90 per cent acetic acid and 0.25 gm. of freshly prepared lead tetraacetate was added. After 2½ hours on the steam bath the mixture was cooled, and 0.13 cc. of concentrated sulfuric acid added; the precipitated solids were removed by centrifuging. The liquid was taken up in ether, washed with water, and the ethereal solution extracted with dilute sodium hydroxide. The alkaline extract was heated for 20 minutes on the steam bath to complete the saponification, then cooled, and acidified. The flocculent precipitate was filtered and dried. Yield 90 mg. of crystals, m.p. 230–237°. After one recrystallization from acetone the material melted at 246–253°.

Neutralization Equivalent—$C_{24}H_{38}O_6$. Calculated, 211.5; found, 209

3(α)-Acetoxy-11‖12-cholane Triacid-11,12,24 (III)—A portion (3.31 gm.) of 3(α)-hydroxy-11‖12-cholane aldehyde 11-diacid-12,24, m.p. 247–253°, dissolved in a mixture of equal volumes of pyridine and acetic anhydride, was heated on the steam bath for 40 minutes, after which the solvent was removed *in vacuo*. The residual oil was dissolved in 53 cc. of acetic acid

and to the cold solution were added 2.31 gm. of chromic acid in 13 cc. of water and 200 cc. of acetic acid. The mixture was placed in a thermostat at 45°. After 4 days the solution was cooled, alcohol added, and the solvent removed under reduced pressure. The residue was extracted with ether and washed with dilute sulfuric acid and with water. Concentration of the dried ethereal solution gave 1.08 gm. of a poorly crystalline product, m.p. 229–232°. Recrystallizations from ether and from ethyl acetate failed to raise the melting point. On concentration of the mother liquor, an additional 0.51 gm. of product of the same melting point was obtained. The total yield of material melting at 229–232° was 42 per cent.

Neutralization Equivalent—$C_{26}H_{40}O_8$. Calculated, 160.2; found, 164

3(α)-Acetoxy-11‖12-cholane Diacid 11,12-Methyl Ester-24 (X)—A solution of 1.08 gm. of the acetoxy triacid, m.p. 229–232°, in 10 cc. of absolute methanol containing 0.1 cc. of concentrated sulfuric acid was allowed to stand overnight at room temperature. The solution was then poured into 150 cc. of cold water and worked up in the usual manner. The resulting oil was acetylated with 2 cc. of acetic anhydride and 2 cc. of pyridine by heating for 20 minutes on the steam bath. An oil was obtained which crystallized readily from dilute methanol to give clusters of needles, m.p. 202–204°. Repeated recrystallizations from dilute methanol gave material which melted constantly at 208–209°. The total yield of crystals melting above 202° was 0.9 gm. (81 per cent).

Analysis—$C_{27}H_{42}O_8$. Calculated, C 65.56, H 8.56; found, C 65.65, H 8.49

3(α)-Acetoxy-11‖12-cholane Diacid Anhydride 11,12-Methyl Ester-24 (XI)—A small amount (0.4 gm.) of the acetoxy diacid methyl ester, m.p. 202–204°, was refluxed for 15 minutes with 7 cc. of pure thionyl chloride. The latter was distilled, 2 cc. of toluene added, and the solution evaporated again to remove traces of thionyl chloride. The residue crystallized almost immediately. Recrystallization from absolute ether gave beautiful needles, m.p. 206–206.5°. The material was neutral and free of halogen. $[\alpha]_D^{17} = +110°$.

Analysis—$C_{27}H_{40}O_7$. Calculated, C 68.04, H 8.46; found, C 68.40, H 8.44

3(α)-Hydroxy-11‖12-cholane Aldehyde 11-Dimethyl Ester-12,24 (XII)—A solution of 0.50 gm. of 3(α)-hydroxy-11‖12-cholane aldehyde 11-diacid-12,24 in ether containing about 20 per cent of methanol was treated with a slight excess of diazomethane in dry ether. After 10 minutes at 25° the solvent was removed and the residue crystallized from ether-pentane. The yield was 0.45 gm. of material melting at 128–129°. After two recrys-

tallizations from ether-pentane the substance melted constantly at 128.5–129.5°. $[\alpha]_D^{17} = +70.4°$.

Analysis—$C_{26}H_{42}O_6$. Calculated, C 69.30, H 9.40; found, C 69.59, H 9.41

3(α)-Acetoxy-11‖12-cholane Aldehyde 11-Dimethyl Ester-12,24 (XIII)—Acetylation of 0.53 gm. of the hydroxy aldehyde dimethyl ester with acetic anhydride and pyridine for 30 minutes at 95° gave a product which crystallized from dilute methanol in a yield of 0.35 gm. (60 per cent), m.p. 125–126°. Recrystallized once from dilute methanol and once from benzene-pentane, it exhibited a constant melting point of 127–128°. $[\alpha]_D^{17} = +94°$.

Analysis—$C_{28}H_{44}O_7$. Calculated, C 68.26, H 9.00; found, C 68.36, H 8.89

3(α)-Acetoxy-11‖12-cholane Aldoxime 11-Dimethyl Ester-12,24 (XVII)—To 0.23 gm. of the acetoxy aldehyde dimethyl ester were added 0.25 gm. of finely powdered hydroxylamine hydrochloride and 5 cc. of pyridine. The material was dissolved by warming and the solution heated overnight on the steam bath. It was then poured into 50 cc. of water and the precipitate extracted with ether. The ethereal solution was washed with water, 5 per cent hydrochloric acid, water, 5 per cent sodium carbonate solution, and water. The ether was removed and the remaining oil (0.20 gm.) dissolved in 5 cc. of methanol. Water was added and the solution boiled until turbid. Very slow cooling gave crystals, m.p. 148–149° (0.12 gm.). One recrystallization from dilute methanol gave crystals which melted constantly at 148.5–149.5°.

Analysis—$C_{28}H_{45}O_7N$. Calculated, C 66.24, H 8.94, N 2.76; found, C 66.53, H 9.06, N 2.77

3(α)-Hydroxy-11‖12-cholane Aldehyde 11-Acid 12-Methyl Ester-24 (XIV)—A solution of 10.0 gm. of the hydroxy aldehyde diacid in 200 cc. of absolute methanol containing 1 cc. of concentrated sulfuric acid was allowed to stand overnight at room temperature. It was then poured into 2 liters of water, extracted with ether, and the ethereal solution washed thoroughly with water and dried over anhydrous magnesium sulfate. On removal of most of the solvent, 8.9 gm. (86 per cent) of the half ester, m.p. 144–147°, crystallized from the solution. Concentration of the mother liquor yielded an additional 0.2 gm. of somewhat less pure material. Recrystallization of the compound from ethyl acetate or absolute ether gave a product melting at 148–149.5°. $[\alpha]_D^{17} = +63.2°$.

Analysis—$C_{25}H_{40}O_6$. Calculated, C 68.77, H 9.24; found, C 68.86, H 9.21

3(α)-Acetoxy-11‖12-cholane Aldehyde 11-Acid 12-Methyl Ester-24 (XV)—Acetylation in the usual manner of 8.4 gm. of 3(α)-hydroxy-11‖12-cholane

aldehyde 11-acid 12-methyl ester-24 led to 8.5 gm. (93 per cent) of the corresponding acetate, m.p. 168–171°, from dilute methanol. $[\alpha]_D^{17} = +82°$.

Analysis—$C_{27}H_{42}O_7$. Calculated, C 67.75, H 8.85; found, C 67.53, H 8.64

Saponification of 3(α)-Hydroxy-11‖12-cholane Aldehyde 11-Dimethyl Ester-12,24 (XII)—(a) A 0.8 gm. portion of the hydroxy aldehyde dimethyl ester was allowed to stand overnight at room temperature with a 5 per cent solution of potassium carbonate in 50 per cent methanol. When the product was worked up, no neutral fraction was observed, but from the dried ethereal solution containing the acid fraction there was obtained a crystalline product, m.p. 157–159°. Several recrystallizations from absolute ether gave material melting at 168–170°.

(b) When 0.10 gm. of the hydroxy aldehyde dimethyl ester was refluxed overnight with 10 cc. of 2 N potassium hydroxide in 75 per cent methanol, the same product was isolated in 70 per cent yield.

(c) About 0.15 gm. of the hydroxy aldehyde dimethyl ester was saponified as in (b), but was worked up in such a manner as to isolate all the organic material in one fraction. This material was thoroughly dried, powdered, and made uniform. A 29.4 mg. sample required 4.62 cc. of 0.0140 N alkali, corresponding to the neutralization value of a monobasic acid.

The product of melting point 168–170°, therefore, was 3(α)-hydroxy-11‖12-cholane aldehyde 11-methyl ester 12-acid-24 (XVI).

3(α)-Acetoxy-11‖12-cholane Aldehyde 11-Acid Chloride 12-Methyl Ester-24 (XVIII)—Exactly 2.00 gm. of 3(α)-acetoxy-11‖12-cholane aldehyde 11-acid 12-methyl ester-24 were dissolved in 10 cc. of pure thionyl chloride and allowed to stand for 30 minutes at room temperature, after which the solution was heated at 50° for 1 hour. The thionyl chloride was removed *in vacuo*, 5 cc. of toluene added, and the solution again evaporated at reduced pressure. The oil was dissolved in 20 cc. of absolute ether under a reflux; on cooling, beautiful needles separated rapidly. These were filtered and washed with a mixture of ether and pentane. The first crop, 1.55 gm. (75 per cent), melted at 138–139°. When pentane was added to the mother liquor, an additional 0.4 gm., m.p. 137–139°, was obtained. Total yield, 94 per cent. The melting point was not lowered by allowing the acid chloride to stand for several days in the open air.

Analysis—$C_{27}H_{41}O_6Cl$. Calculated, Cl 7.13; found, Cl 8.69

Action of Methanol on Acid Chloride—A portion (0.10 gm.) of the above acid chloride was refluxed overnight with 10 cc. of absolute methanol. Then 0.5 cc. of concentrated sulfuric acid was added and the refluxing continued for 2 hours. The solution was poured into water, extracted with ether, and the ethereal solution washed with water, 5 per cent potassium

carbonate solution, and water. Removal of the ether and evaporation of the residue with benzene gave 0.06 gm. of neutral fraction. This was readily crystallized, by dissolving in ether and adding pentane, to yield 3(α)-hydroxy-11‖12-cholane aldehyde 11-dimethyl ester-12,24, m.p. 129–130°.

Action of Diazomethane on Acid Chloride—(a) A solution of 1.0 gm. of the acid chloride, m.p. 138–139°, in 10 cc. of toluene was added to a cold (0–5°) dried solution of diazomethane (prepared from 4 gm. of methylnitrosourea) in about 200 cc. of ether. After standing for 2 hours, the solvent was removed under reduced pressure to give an oil which soon crystallized completely. The material, recrystallized once from ether-pentane, melted at 138–139°, and a mixed melting point determination with starting material showed no depression.

(b) To a dry solution of the diazomethane from 1 gm. of methylnitrosourea in 100 cc. of ether was added 0.25 gm. (5.0 $\times$ 10^{-4} mole) of the acid chloride dissolved in 20 cc. of absolute ether. The solution was refluxed for 24 hours. At the end of this time it was still light yellow in color and contained a small amount of polymeric material, presumably formed by the action of traces of water on the diazomethane. The ether was removed and the residue refluxed for an hour with 20 cc. of a 10 per cent solution of sodium hydroxide in 50 per cent methanol. Water was added and most of the liquid distilled to remove methanol. The remaining solution was cooled, an excess of nitric acid added, and the precipitated organic acid filtered. The filtrate was analyzed for chloride ion by the Volhard method. It contained 5.2 $\times$ 10^{-4} mole of chloride.

(c) A dried solution of the diazomethane from 1 gm. of methylnitrosourea in 40 cc. of ether was added to 150 cc. of pure anhydrous dioxane. Into this was poured a solution of 94.7 mg. (1.91 $\times$ 10^{-4} mole) of the acid chloride in 10 cc. of dioxane, and the solution (in a closed pressure flask) was left for 24 hours in a thermostat at 50°. After 12 hours the yellow color of diazomethane was still present, but it had disappeared at the end of 24 hours. The material was worked up as in (b). The filtrate, after treatment with alkali, acidification, and removal of insoluble organic material, contained 1.74 $\times$ 10^{-4} mole of chloride ion, corresponding to a loss of 9 per cent of the original chlorine. This small loss of chlorine may be explained by evolution of hydrogen chloride formed on hydrolysis of the acid chloride by traces of water which must inevitably have been present.

Action of Pyridine on Acid Chloride. Preparation of Compound A— A portion of the acid chloride (2.3 gm.) was dissolved in 50 cc. of pyridine which had been distilled over potassium hydroxide. After 5 days at 45°, the solvent was removed, leaving a yellow, oily residue. Crystallization occurred on addition of toluene. The toluene was removed *in vacuo*.

Treatment with either dioxane or ether left an insoluble residue. A small fraction was removed, washed three times with ether by centrifuging, and dried in a stream of air. The product, a yellow powder, melted unsharply at 195°. It gave a strong Beilstein test.

The bulk of the material was triturated with 60 cc. of anhydrous ether, filtered, washed with ether, and dried in a vacuum desiccator over concentrated sulfuric acid. The yield was 1.5 gm. of a pinkish colored powder. Concentration of the filtrate gave 0.3 gm. of white crystals, m.p. 192–197°, and a negative Beilstein test. After two recrystallizations from methanol this product, Compound A, melted at 199–201°. $[\alpha]_D^{17} = +123°$.

When 0.8 gm. of the powder, m.p. 195°, was added in several portions to a cold (10°) ethereal solution of the diazomethane from 5 gm. of methylnitrosourea, vigorous gas evolution was observed after each addition, and a white material appeared immediately at the bottom of the flask. After standing overnight in the ice box, the solid material was filtered and washed with absolute ether. The crude product melted at 192–197°. After several recrystallizations from methanol and from benzene-pentane it melted at 200–201°. $[\alpha]_D^{17} = +122°$.

When 50 mg. of the same pinkish colored powder were refluxed for 20 minutes in 50 cc. of absolute ether, a small light colored residue remained undissolved. This was filtered off and identified as pyridine hydrochloride by its ready solubility in water to give a solution containing chloride ion. The ethereal filtrate on concentration to a small volume yielded crystals melting at 189–196°; recrystallized from methanol, the material melted at 195–199°. A mixed melting point determination with Compound A gave a value of 199–201°.

Determination of Structure of Compound A. Properties—M.p. 199–201°; fine white powder; $[\alpha]_D^{17} = +122°$. Sparingly soluble in ether, soluble in benzene, acetone, hot methanol. Chromatographically homogeneous; eluted from alumina by 99 per cent ether-1 per cent acetone. Neutral. The material generally decomposed during the course of cryoscopic molecular weight determinations in camphor, but one apparently satisfactory determination gave a value of 948. Sufficient material was not available to permit an accurate cryoscopic determination in benzene.

Analytical—The compound does not contain nitrogen or halogen.

$C_{54}H_{82}O_{13}$ (XXI).   Calculated, C 69.05, H 8.80; found, C 69.27, H 8.58

Acetylation—100 mg. of Compound A were treated with acetic anhydride and pyridine in the usual manner. When it was worked up, unchanged starting material was recovered almost quantitatively.

Treatment with Absolute Methanol Containing 2 Per Cent Sulfuric Acid—Exactly 73 mg. of Compound A were refluxed for 2 hours with 10 cc. of

absolute methanol containing 0.1 cc. of concentrated sulfuric acid. The solution was cooled, poured into 100 cc. of water, and extracted with ether. Washing with dilute carbonate gave an acid fraction, which weighed 38 mg. The neutral fraction, in ether, was dried and concentrated to about 3 cc., and 1 cc. of pentane was added. Long needles separated immediately, m.p. 129–131°. After one recrystallization from ether-pentane the material melted at 130.5–131° and showed no depression of the melting point when mixed with an authentic sample of $3(\alpha)$-hydroxy-11‖12-cholane aldehyde 11-dimethyl ester-12,24 (XII).

Treatment with Hydroxylamine—To a solution of 106 mg. of Compound A in 7 cc. of boiling methanol was added a solution of 0.1 gm. of hydroxylamine and 0.1 gm. of sodium acetate in 3 cc. of water. After refluxing overnight, the reaction mixture was worked up as usual to yield a 33 mg. acid fraction and an 80 mg. neutral fraction. The acid fraction was not esterified by refluxing for 2 hours with absolute methanol containing 2 per cent concentrated sulfuric acid, indicating that the quaternary Ring C carboxyl group was present. The 80 mg. of neutral fraction on being chromatographed over alumina gave 54 mg. of a material which readily crystallized from benzene-pentane, m.p. 181.5–183°.

Analysis—$C_{54}H_{84}O_{13}N_2$ (dioxime of XXI). Calculated. C 66.91, H 8.74, N 2.89
Found. " 66.78, " 8.76, " 3.17

SUMMARY

Two methods are described for opening Ring C in desoxycholic acid without affecting the unprotected hydroxyl group in Ring A. The product is $3(\alpha)$-hydroxy-11‖12-cholane aldehyde 11-diacid-12,24, m.p. 248–254°.

Various derivatives of the resulting compound have been prepared and characterized. They are $3(\alpha)$-acetoxy-11‖12-cholane aldehyde triacid-11,12,24, m.p. 247–253°; $3(\alpha)$-acetoxy-11‖12-cholane diacid 11,12-methyl ester-24, m.p. 208–209°; $3(\alpha)$-acetoxy-11‖12-cholane diacid anhydride 11,12-methyl ester-24, m.p. 206–206.5°; $3(\alpha)$-hydroxy-11‖12-cholane aldehyde 11-dimethyl ester-12,24, m.p. 128.5–129.5°; $3(\alpha)$-acetoxy-11‖12-cholane aldehyde 11-dimethyl ester-12,24, m.p. 127–128°; $3(\alpha)$-acetoxy-11‖12-cholane aldoxime 11-dimethyl ester-12,24, m.p. 148.5–149.5°; $3(\alpha)$-hydroxy-11‖12-cholane aldehyde 11-acid 12-methyl ester-24, m.p. 148– ·

149.5°; 3(α)-acetoxy-11‖12-cholane aldehyde 11-acid 12-methyl ester-24, m.p. 168–171°; 3(α)-hydroxy-11‖12-cholane aldehyde 11-methyl ester 12-acid-24, m.p. 168–170°; and 3(α)-acetoxy-11‖12-cholane aldehyde 11-acid chloride 12-methyl ester-24, m.p. 138–139°.

It has been shown that a compound with an acid chloride group attached to the quaternary C_{13}-carbon atom in the steroid nucleus will not react with diazomethane under the conditions employed.

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EXPERIMENTAL STUDIES IN THE STEROIDS. A NOVEL METHOD FOR THE PREPARATION OF STEROL DICHLORIDES*

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As is well known, the sterols are monohydric polycyclic secondary alcohols, many of which are unsaturated at the 5,6 position. The various sterols differ in the number of double bonds and in the structure of the side chain.

(I)

One of the more important reactions in sterol chemistry is the temporary inactivation of the double bond in the 5,6 position. Permanent inactivation is generally brought about by hydrogenation (1). Temporary inactivation has been effected by the addition of bromine (2) or by conversion to the i-ethers ((3), cf. (4)). In the case of free bromine, the reaction is very rapid and the yield of dibromide is generally good. That this is a temporary protection of the double bond is shown by the fact that zinc and acetic acid will remove the bromine atoms.

With free chlorine the results of the reaction are very different. Most of the product is oily and chlorine is apt to enter into many places in the sterol molecule. The desired crystalline dichloride is hard to obtain and the yield is very poor. It should be noted, however, that in many cases the dichloride would be preferable for synthetic work, since it is relatively more stable. It is our purpose in this paper to discuss the results of experi-

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ments designed to make more readily available in better yields these di-
chlorides of the sterols.

In 1938 it was first shown by Wallis and Mead[1] that iodobenzene
dichloride could be successfully used temporarily to protect the double bond
in cholesterol by the addition of chlorine. The cholesterol dichloride so
obtained was very easily prepared in excellent yield. We have con-
tinued the study of this reaction because its practicality was apparent from
the first experiments. Our results show that under a variety of conditions
the reaction can be carried out with no difficulties and that excellent yields
of desired products can be obtained. Unlike the reaction with free bromine

(II)   (III)

(IV)   (V)

or free chlorine, this method of addition of chlorine to the double bond
produces two isomeric dichlorides. Theoretically, of course, the possi-
bility of four different isomeric dichlorides exists. However, until this
reaction was studied, only one of the possible isomers had been obtained.

It is of interest to note that the isomeric dichlorides obtained by this
method differ greatly in their physical properties, such as melting point,
solubility, specific rotation, etc. In this paper there are described the
results of our experiments on the action of iodobenzene dichloride on cho-

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[1] The experimental details of this reaction are taken from a senior thesis sub-
mitted by J. F. Mead in partial fulfilment of the requirements for the degree of
Bachelor of Arts, Princeton University, 1938.

lesteryl benzoate (II), cholesteryl p-toluenesulfonate (III), β-sitosteryl benzoate (IV), and stigmasteryl benzoate (V). In each case two isomeric dichlorides were obtained. With cholesteryl benzoate, the dichloride of lower melting point was shown to be identical with the benzoate dichloride prepared from the cholesterol dichloride of Mauthner and Suida (5). In our studies on stigmasteryl benzoate, only 1 mole of iodobenzene dichloride was used, and no difficulties were observed in the selective chlorination of the double bond in the 5,6 position. In Table I are listed the melting points and rotations of the various dichlorides which we have prepared by this method.

We should now like to point out that certain fundamental differences in the chemical properties of these isomeric dichlorides have been observed. For example, when the above benzoate dichlorides were subjected to hydrolysis by refluxing with alcoholic potassium hydroxide, the isomer of lower

TABLE I

Starting material	Melting point of dichlorides	Specific rotation
	°C.	degrees
Cholesteryl benzoate	251	+13.4
	120	−20.2
" p-toluenesulfonate	191	−6.1
	85	−39.0
β-Sitosteryl benzoate	222	+14.6
	131.5	−17.1
Stigmasteryl "	235	−13.0
	145	−33.8

melting point always gave in good yields the corresponding sterol dichloride. The higher melting isomer, however, in each case gave one of a new series of compounds, which were found to be monochloro derivatives. Cholesteryl benzoate dichloride, m.p. 251°, yields 6-chlorocholesterol, m.p. 152°. Its corresponding acetate melts at 130°, and its benzoate melts at 205°.

(VI)   (VII)

Certain other interesting properties of these compounds should be noted. When a pair of these benzoate dichlorides is refluxed with zinc and acetic acid, both isomers give the sterol benzoate. This fact was checked many times by melting point, mixed melting point, and saponification to the sterol.

The tosylates of the dichlorides have been found to be peculiarly resistant to hydrolysis, it being impossible to convert either isomer to its corresponding sterol dichloride by the usual hydrolytic reagents.

In the description of our results outlined above, it has been noted that when the high melting isomer of cholesterol benzoate dichloride is hydrolyzed 6-chlorocholesterol is obtained. It is possible to formulate the structure of this compound in two ways. The high negative rotation of our 6-chloro compound suggests strongly that the double bond is in the 5,6 position (6). Further evidence of this is offered by a comparison with the physical properties of 6-iodocholesterol prepared by Levin and Spielman (7) using a totally different method (see Table II).

$$\xrightarrow[\mathrm{Hg\,(CN),NaCl}]{\mathrm{Hg\,(OAc)_2}}$$

$$\xrightarrow{\mathrm{I_2}}$$

HgCl

I

TABLE II

	6-Chlorocholesterol	6-Iodocholesterol
	°C.	°C.
Free sterol.....................	152	158
Acetate........................	130	116
Benzoate.......................	205	215

Scheme A

In this connection it is of interest to note that Spring and Swain (8) have reported the preparation of the benzoate of compound VII. We would like to point out that a study of the physical and chemical properties of their compound, reported by them in a later paper (9), leads us to doubt the correctness of their formulation. For instance, their compound has a

high negative rotation, $[\alpha]_D = -79°$. The melting point recorded by them is 127°, differentiating it sharply from our 6-chlorocholesteryl benzoate. It seems to us that the principal doubt on the structure assigned by them derives from the fact that dehalogenation of their compound with anhydrous potassium acetate in absolute alcohol leads to the formation of a compound of known structure, Δ-cholestenediol-3,4-benzoate-4 (A). Although an explanation (Scheme A) for this peculiar reaction is offered by Rosenheim (10), we are of the opinion that the structure of the compound prepared by Spring and Swain most probably has the formula shown in B and that it is formed according to the sequence of reactions presented in Scheme B.

EXPERIMENTAL

Cholesterol Dichloride[1]—To 2.45 gm. of carefully dried cholesterol in a solution of dry chloroform were added 1.93 gm. of iodobenzene dichloride prepared according to the method of Willgerodt (11). The mixture was refluxed for about half an hour after the disappearance of the crystals of iodobenzene dichloride. The chloroform was then distilled *in vacuo*, and the residue recrystallized several times from ethyl alcohol. The yield of cholesterol dichloride, m.p. 132–135°, was 2.35 gm. (81 per cent).

β-Sitosteryl Benzoate Dichloride—To 2 gm. of β-sitosteryl benzoate dissolved in 50 cc. of chloroform were added 1.8 gm. of iodobenzene dichloride. The mixture was kept at 40° for half an hour. The temperature was then raised to the boiling point of chloroform and all the latter was removed on the water bath. The product was taken up in warm ethyl alcohol. Crystals appeared almost at once. They were filtered. Yield of crude material, 0.95 gm., m.p. 199–201°. After three recrystallizations from a mixture of benzene and alcohol, long, filamentous crystals were obtained which melted at 220–222°; $[\alpha]_D^{17} = +14.6°$ (20 mg. in 2 cc. of dry chloroform; length of tube, 1 dm.).

Scheme B

Analysis—$C_{36}H_{54}O_2Cl_2$.　Calculated.　C 73.36, H 9.18, Cl 11.96
　　　　　　　　　　　　Found.　　" 72.94, " 0.97, " 12.8

The filtrate from the first crude crystalline material was diluted with water and placed in the ice box. A crystalline product (0.60 gm.) was obtained, m.p. 120–125°. Four recrystallizations of this material from a mixture of acetone and methanol gave a product which melted at 130–131.5°; $[\alpha]_D^{17} = -17.1°$ (1 dm. tube; 20 mg. in 2 cc. of chloroform solution).

Analysis—$C_{36}H_{54}O_2Cl_2$.　Calculated.　C 73.36, H 9.18, Cl 11.96
　　　　　　　　　　　　Found.　　" 73.33, " 9.15, " 12.19

The higher melting isomer is soluble in benzene and chloroform, but is not appreciably soluble in most other solvents. The lower melting isomer is soluble in benzene, chloroform, ethyl acetate, and acetone. It is insoluble in methyl and ethyl alcohols.

On dehalogenation with zinc and acetic acid for 6 hours, both isomers give β-sitosteryl benzoate, m.p. 146°. The product so obtained gives no depression of the melting point with the starting material. The dehalogenated product from the lower melting isomer on saponification gave pure β-sitosterol, m.p. 136°.

Cholesteryl p-Toluenesulfonate Dichloride—To 2.6 gm. of cholesteryl p-toluenesulfonate dissolved in 50 cc. of chloroform were added 1.4 gm. of iodobenzene dichloride. The suspension was allowed to stand for 1 hour at 20°. It was then warmed to 45° until the iodobenzene dichloride crystals dissolved. The solution was refluxed for 1 minute. It was then allowed to cool and the chloroform was evaporated at room temperature. The residue was taken up in pentane and crystallized. Yield of crude product, 1.3 gm. When the filtrate was cooled in the ice box at 0°, an additional 0.5 gm. of product was obtained. When recrystallized from benzene and petroleum ether followed by recrystallization from acetone-alcohol, the product melted at 190–191°; $[\alpha]_D^{17} = -6.1°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{34}H_{52}O_3SCl_2$.　Calculated.　C 66.76, H 8.57, Cl 11.59
　　　　　　　　　　　　Found.　　" 66.69, " 8.60, " 11.86

The original filtrate from the pentane crystallization gave an oil on evaporation. This was triturated with methyl alcohol to remove iodobenzene. The methyl alcohol was then removed by water. Weight of dried product, 1.0 gm. This material was dissolved in acetone and crystallized from a mixture of acetone and alcohol. A second recrystallization gave a crystalline product, m.p. 84–85°; $[\alpha]_D^{17} = -39°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{34}H_{52}O_4SCl_2$. Calculated. C 66.76, H 8.57, Cl 11.59
Found.   " 66.79, " 8.66, " 11.31

The high melting isomer, m.p. 190–191°, was taken up in acetone and boiled with dilute hydrochloric acid for 1 hour in an effort to remove the *p*-toluenesulfonate group. The product was recovered, however, unchanged. Portions of both isomers were treated with potassium hydroxide in alcohol with no apparent hydrolysis taking place. Similar results were obtained with the high melting isomer when concentrated hydrochloric acid in dioxene was used, and when sodium methylate in benzene solution was employed as the hydrolytic agent.

Cholesteryl Benzoate Dichloride—To 5 gm. of cholesteryl benzoate dissolved in chloroform were added 2.9 gm. of iodobenzene dichloride. The mixture so obtained was treated in the manner described above, and on removal of the chloroform a crystalline product was obtained, m.p. 244–247°. Recrystallization from ethyl alcohol gave crystals melting at 250–251°; $[\alpha]_D^{25} = +13.4°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{34}H_{50}O_2Cl_2$. Calculated. C 72.11, H 9.17, Cl 12.90
Found.   " 72.26, " 9.07, " 12.63

The mother liquors were evaporated and taken up in methyl alcohol. A small amount of water was added and the mixture was set aside for crystallization. Crystals were obtained which melted unsharply at about 110°. When recrystallized from ethyl acetate-methanol solution, the product melted at 117–120°, and had $[\alpha]_D^{25} = -20.2°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{34}H_{50}O_2Cl_2$. Calculated. C 72.11, H 9.17, Cl 12.90
Found.   " 72.70, " 9.18, " 12.43

The high melting isomer, m.p. 250–251°, was treated on the water bath with an excess of potassium hydroxide dissolved in ethyl alcohol. After 15 minutes the solid had gone into solution and the mixture began to turn slightly yellow. The reaction mixture was worked up at this point. The material was poured into water and extracted with ether. From the ether solution a crystalline residue was obtained. Recrystallization from ethyl alcohol gave a product of melting point 150–152°, $[\alpha]_D^{18} = -63°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube), which we believe to be 6-chlorocholesterol.

Analysis—$C_{27}H_{45}OCl$. Calculated. C 77.00, H 10.77, Cl 8.43
Found.   " 77.1, " 10.7, " 8.57

A portion of this material, 0.5 gm., when treated with acetic anhydride for 1 hour and worked up in the usual manner, gave a crystalline acetate,

m.p. 128–130°; $[\alpha]_D^{27} = -41.2°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{29}H_{47}O_2Cl$. Calculated, C 75.19, H 10.23; found, C 75.24, H 10.37

Another portion of 6-chlorocholesterol when treated with benzoyl chloride in pyridine gave a benzoate melting at 205°; $[\alpha]_D^{27} = -20.4°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Stigmasteryl Benzoate Dichloride—To 0.95 gm. of stigmasteryl benzoate dissolved in 35 cc. of chloroform was added 0.50 gm. of iodobenzene dichloride. When the reaction was carried out in a manner similar to that described above, a residue was obtained which on crystallization from benzene and ethyl alcohol gave 0.3 gm. of fluffy crystals, m.p. 221–224°. Recrystallization gave a product, m.p. 234–235°; $[\alpha]_D^{17} = -13.0°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{36}H_{52}O_2Cl_2$. Calculated. C 73.57, H 8.92, Cl 12.06
Found.   " 73.41, " 8.99, " 12.04

The mother liquors obtained from the first crystallization gave a fluffy precipitate, which was filtered. The residual solution was evaporated to dryness and the oily residue was taken up in acetone. On addition of a drop of water and a small amount of methyl alcohol small needles separated from the cold solution, m.p. 130–143°; weight of the crude product, 0.15 gm. Further treatment of the mother liquors with a small amount of water and methyl alcohol gave additional crystals, m.p. 132–140°. These two fractions were united and recrystallized from acetone-methyl alcohol. Yield of crystalline product, 0.15 gm., m.p. 137–145°. When recrystallized, the material melted at 144–145°; $[\alpha]_D^{17} = -33.8°$ (20 mg. in 2 cc. of chloroform solution; 1 dm. tube).

Analysis—$C_{36}H_{52}O_2Cl_2$. Calculated. C 73.57, H 8.92, Cl 12.06
Found.   " 73.67, " 8.91, " 12.11

SUMMARY

A novel method for the preparation of sterol ester dichlorides involving the use of iodobenzene dichloride as a halogenating agent has been de-

scribed. Each sterol ester used gives two isomeric dichloro compounds, differing in physical and chemical properties. The following pairs of dichloro compounds were made: cholesteryl benzoate dichlorides of melting points 120° and 251°, cholesteryl p-toluenesulfonate dichlorides of melting points 85° and 192°, β-sitosteryl benzoate dichlorides of melting points 130.5° and 222°, and stigmasteryl benzoate dichlorides of melting points 145° and 235°.

The preparation of 6-chlorocholesterol from the cholesteryl benzoate dichloride of melting point 251° is described. It has a melting point of 152°. Its acetate has a melting point of 130°, and its benzoate 205°.

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THE BROMINE DEGRADATION OF THE SILVER SALTS OF BILE ACIDS AND RELATED COMPOUNDS*

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The reaction of bromine with the silver salt of a carboxylic acid to yield an alkyl bromide containing 1 less carbon atom

$$RCO_2Ag + Br_2 \rightarrow R \cdot Br + CO_2 + AgBr$$

has recently been studied by Hunsdiecker and Hunsdiecker (1).[1] In the present paper are reported the results obtained by application of this reaction to some bile acids and related compounds.

At the beginning of this work we had three principal objectives in mind. First, we wished to study the possibility of developing a method of degrading the bile acid side chain 2 carbon atoms at a time. Such a method would involve conversion of the silver salt of a bile acid to a norcholanyl bromide, dehydrobromination to a Δ^{22}-norcholene, followed by ozonolysis leading to a bisnorcholanic acid. Secondly, we desired to use the alkyl bromides

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[1] In this article (1) will be found reference to the earlier literature on the reaction.

produced in this way as intermediates in the preparation of steroids containing a simple secondary alkyl group at C_{17}. Such molecules might well be better suited for Ruzicka type oxidations (*cf.* (2)) for the removal of the side chain than the carboxylic acids, esters, diphenylethylene, or diphenylethane derivatives which have been studied. Finally, we were

particularly interested in preparing derivatives of the isomeric 20-bromo-pregnanes, since these, on dehydrobromination, would be expected to give $\Delta^{17,20}$ - or $\Delta^{20,21}$ - pregnenes, or both. The former on ozonolysis should yield an etiocholanone-17, and the latter an etiocholanecarboxylic acid. Both types of compounds are of importance as intermediates in the synthesis of the adrenal hormones.

Silver $3(\alpha),12(\beta)$-diacetoxycholanate (I) was prepared in essentially quantitative yield by treating a solution of the sodium salt of desoxycholic acid diacetate in dilute alcohol with an excess of aqueous silver nitrate solution. Upon addition of 1.06 moles of bromine to a suspension of the thoroughly dried silver salt in boiling carbon tetrachloride, vigorous carbon dioxide evolution was observed, and silver bromide appeared. When the reaction mixture was worked up, $3(\alpha),12(\beta)$-diacetoxy-23-bromonorcholane (II), m.p. 127.5–128.5°, $[\alpha]_D^{20} = +114°$, was obtained in 25 per cent yield. In like manner the silver salt of nordesoxycholic acid diacetate (III) was

converted to $3(\alpha),12(\beta)$-diacetoxy-22-bromobisnorcholane (IV), m.p. 144.5–145°, $[\alpha]_D^{20} = +107°$, in 40 per cent yield. When applied to the silver salt of bisnordesoxycholic acid diacetate (V), the bromine degradation led to a mixture of two isomeric bromides which was separated by chromatographic analysis and fractional crystallization. The more easily purified compound, designated $3(\alpha),12(\beta)$-diacetoxy-20(β)-bromopregnane (VI), crystallized in the form of small, hard needles, melting at 140–142°, $[\alpha]_D^{20} = +86°$. The isomeric $3(\alpha),12(\beta)$-diacetoxy-20(α)-bromopregnane (VII) appeared as fine, silky needles, m.p. 83–87°, $[\alpha]_D^{20} = +103°$. The yield of crystalline C_{20}-bromide mixture, before separation, was 65 per cent.

It was to be expected that a ketone group in the molecule would not interfere with the reaction, provided that no excess of bromine was used, since the silver salts react véry rapidly with the halógen. This proved to be the case. From silver $3(\alpha)$-acetoxy-12-ketocholanate (VIII) the corresponding bromide, $3(\alpha)$-acetoxy-12-keto-23-bromonorcholane (IX), m.p. 209.5–211°, $[\alpha]_D^{20} = +126°$, was prepared in 60 per cent yield.

The yields of the bromides as given above are those actually obtained in typical experiments. However, with all of the compounds except des-

CH₃
O   CH—CH₂—CH₂—CO₂—Ag

Br₂

OAc

(VIII)

CH₃
O   CH—CH₂—CH₂—Br

+ CO₂ + AgBr

OAc

(IX)

oxycholic acid, varying amounts of the acetylated acids were recovered from the acid fractions. Correction for this recovery of starting material increases the yields cited above from 5 to 25 per cent.

With the alkyl bromide derivatives described above, reduction with zinc and acetic acid proceeded smoothly to give the corresponding steroids with alkyl side chains in yields which were uniformly over 90 per cent. The compounds so prepared are $3(\alpha),12(\beta)$-diacetoxynorcholane (X), m.p. 116.5–117°, $[\alpha]_D^{20} = +118°$; $3(\alpha),12(\beta)$-diacetoxybisnorcholane (XI), m.p. 115–116°, $[\alpha]_D^{20} = +104°$; $3(\alpha),12(\beta)$-diacetoxypregnane (XII),

m.p. 158–158.5°, $[\alpha]_D^{20} = +112°$; and 3(α)-acetoxy-12-ketonorcholane (XIII), m.p. 158.5–159.5°, $[\alpha]_D^{20} = +119°$.

Attempts to remove hydrogen bromide from the two primary diacetoxy bromides (II and IV) were unsuccessful. When the bromides were refluxed with collidine, unchanged starting material was recovered; treatment with piperidine led to stable piperidinium complexes. In the case of 3(α),-12(β)-diacetoxy-23-bromonorcholane, treatment with sodium ethylate, followed by reacetylation, gave in very low yield a compound melting at

(X)

(XI)

(XII)

(XIII)

143–144°, $[\alpha]_D^{20} = +60°$, which on analysis proved to be acetoxy-23-ethoxy-norcholene. This apparently results from simultaneous ether formation and loss of a molecule of acetic acid from either Ring A or Ring C. The compound was not further investigated. Treatment of the primary bromides with alcoholic silver nitrate solution led to precipitation of silver bromide, but no definite organic product could be isolated.

The mixture of secondary C_{20}-bromides proved stable toward collidine, but when it was refluxed for 22 hours with piperidine, hydrogen bromide was removed and a product melting at 162–174° was isolated in 87 per cent yield by crystallization from methanol. After several recrystallizations,

material with a constant melting point of 176–178.5°, $[\alpha]_D^{20} = +112°$, was obtained. The product analyzed correctly for a diacetoxypregnene, and is presumably $3(\alpha),12(\beta)$-diacetoxy-$\Delta^{17,20}$-pregnene (XIV), $3(\alpha),12(\beta)$-diacetoxy-$\Delta^{20,21}$-pregnene (XV), or a mixture of both.

Ozonolysis of our material, or preferably of the related derivative or mixture prepared from $3(\alpha)$-acetoxy-11-keto-20-bromopregnane, should lead to a C_{17}-keto compound, an etio acid, or a readily separable mixture of the two.

CH₃ | CHBr — AcO —

AcO

CH₃ | CH — AcO — and (or)

AcO

(XIV)

CH₂ ‖ CH — AcO —

AcO

(XV)

EXPERIMENTAL[2]

$3(\alpha),12(\beta)$-*Diacetoxy-23-bromonorcholane* (*II*)—Desoxycholic acid diacetate was prepared by heating 24.8 gm. of desoxycholic acid on the steam bath for 3 hours with 50 cc. of pyridine and 45 cc. of acetic anhydride. The product was worked up in the usual manner and the oil so obtained was dissolved in 300 cc. of 80 per cent ethyl alcohol. This solution was titrated to the phenolphthalein end-point with dilute aqueous sodium hydroxide, after which the pink color was just discharged by the dropwise addition of very dilute nitric acid.

[2] All melting points are uncorrected. Unless otherwise noted, rotations were taken in acetone in a 1 dm. tube ($c = 0.8$ to 1.3).

The solution was diluted to 800 cc. by addition of water, and a solution of 12.9 gm. (20 per cent excess) of silver nitrate in 150 cc. of water was added with swirling. About 500 cc. of water were added, and after thorough shaking the voluminous white precipitate was filtered off, washed three times with 20 per cent ethyl alcohol, and dried to constant weight, finally at 100° and 1 mm. over phosphorus pentoxide. The yield of light tan, powdery silver salt was 90 per cent.

To a suspension of 23.8 gm. of silver salt in 500 cc. of boiling anhydrous carbon tetrachloride were added over a period of about 10 seconds and with vigorous stirring 6.92 gm. (1.06 moles) of pure bromine in 147 cc. of carbon tetrachloride. Evolution of carbon dioxide and formation of silver bromide were observed immediately, and the color of bromine was discharged in the course of 4 minutes.

The stirring and refluxing were continued for an additional 10 minutes. The hot suspension was filtered to remove silver bromide and the solvent was taken off *in vacuo* below 40°. The light yellow residual oil was taken up in ether and the ethereal solution washed three times with 5 per cent sodium carbonate and three times with water. After the mixture had dried over sodium sulfate, the ether was removed to yield 10.6 gm. of a neutral, red oil. The oil was chromatographed on alumina. Fractions eluted by benzene and by benzene containing 1 per cent of ether crystallized on removal of the solvent and addition of methanol, m.p. 124.5–127°. Yield (combined fractions from several chromatographic analyses) 5.2 gm. (25 per cent). One recrystallization from methanol gave needles melting at 127.5–128.5°. The melting point was unchanged by further recrystallization. $[\alpha]_D^{20} = +114°$.

Analysis—$C_{27}H_{43}O_4Br$.   Calculated.   C 63.39, H 8.47, Br 15.62
                Found.   " 63.65, " 8.55, " 15.93

3(α),12(β)-Diacetoxy-22-bromobisnorcholane (*IV*)—Pure crystalline nordesoxycholic acid diacetate, m.p. 205–206°, was converted to the silver salt by the method described above. A 0.90 gm. portion of the salt, suspended in boiling carbon tetrachloride, was treated with 0.268 gm. (1.06 moles) of bromine in 1 cc. of carbon tetrachloride. After 10 minutes the reaction mixture was worked up as before, yielding a neutral fraction of 0.489 gm. and an acid fraction of 0.26 gm. The neutral fraction, upon being chromatographed on alumina, gave 0.30 gm. of needles, m.p. 143–144°. After one recrystallization from methanol, the product melted constantly at 144.5–145°; $[\alpha]_D^{20} = +107°$

Analysis—$C_{26}H_{41}O_4Br$.   Calculated.   C 62.76, H 8.31, Br 16.06
                Found.   " 62.85, " 8.59, " 16.68

The acid fraction was crystallized and identified by melting point and mixed melting point as nordesoxycholic acid diacetate.

Isomeric 3(α),12(β)-Diacetoxy-20-bromopregnanes (VI and VII)—From 2.77 gm. of non-crystalline bisnordesoxycholic acid diacetate were pre-prepared by the usual method 3.48 gm. (83 per cent) of its silver salt. This, suspended in carbon tetrachloride, was treated with bromine and worked up as previously indicated. The neutral fraction weighed 1.88 gm. and the acid fraction 1.11 gm.

Two-thirds (1.2 gm.) of the neutral fraction, dissolved in 20 cc. of benzene plus 80 cc. of petroleum ether, was chromatographed on 40 gm. of alumina. Elutions with mixtures of benzene and petroleum ether gave in the first fraction 953 mg. of fine, yellowish needles. The second fraction, when eluted by benzene containing a few per cent of ether, gave 101 mg. of coarse needles.

The material in the second fraction was twice recrystallized from methanol to give small, hard needles, m.p. 140–142°; $[\alpha]_D^{20} = +86°$ (designated as the 20(β)-bromo compound).

Analysis—$C_{25}H_{39}O_4Br$. Calculated, C 62.10, H 8.13; found, C 61.73, H 8.22

The first fraction on crystallization from methanol gave material melting at 103–106°. By addition of water to the mother liquor, 0.10 gm. of fine, silky needles, m.p. 83–87°, was obtained. The melting point remained constant on further recrystallization. $[\alpha]_D^{20} = +103°$. (This isomer has been designated the 20(α)-bromo compound.)

Analysis—$C_{25}H_{39}O_4Br$. Calculated, C 62.10, H 8.13; found, C 61.73, H 8.19

The material melting at 103–106°, mentioned above, had a specific rotation of $[\alpha]_D^{20} = +97°$. This intermediate rotation indicates that the first material obtained from Fraction I was a mixture of the two isomers.

3(α)-Acetoxy-12-keto-23-bromonorcholane (IX)—To 5.1 gm. of the silver salt of 3(α)-acetoxy-12-ketocholanic acid in 75 cc. of boiling carbon tetra-chloride was added a solution of 1.52 gm. (1.00 mole) of bromine in 4.85 cc. of carbon tetrachloride. When, after filtration of the silver bromide, the solvent was removed *in vacuo*, the residue crystallized. It was broken up, filtered, and washed with ether. In this manner 1.61 gm. of crystals, m.p. 190–205°, were obtained. Recrystallization from chloroform-methanol gave 1.18 gm. of material, m.p. 205–208°. Two more recrystallizations from chloroform-methanol and two from dilute acetone gave a pure product, m.p. 209.5–211°. $[\alpha]_D^{20} = +126°$ (chloroform).

Analysis—$C_{25}H_{39}O_3Br$.   Calculated.   C 64.23, H 8.41, Br 17.1
                                 Found.   " 64.27, " 8.63, " 16.4

When reduced with zinc and acetic acid, 0.36 gm. of the bromo compound gave 0.29 gm. (97 per cent) of 3(α)-acetoxy-12-ketonorcholane (XIII), m.p. 157–159°. After two recrystallizations from methanol, the product melted at 158.5–159.5°. $[α]_D^{20} = +119°$.

3(α),12(β)-Diacetoxynorcholane (X)—A portion of 3(α),12(β)-diacetoxy-23-bromonorcholane (4.48 gm.) was dissolved in about 150 cc. of glacial acetic acid and to the refluxing solution were added portionwise over a period of 15 minutes about 6 gm. of zinc dust. The hot solution was filtered directly into 5 volumes of cold water, and the residual zinc washed thoroughly with ether. The precipitated product was removed from the water by extraction with ether. The ethereal solution was washed with water, three times with 5 per cent sodium carbonate solution, and three times with water. The solution was dried over sodium sulfate and the ether removed to give 3.7 gm. of colorless oil. This was crystallized from methanol. The first crop of fine, long needles weighed 1.95 gm., m.p. 112–114°; the Beilstein test was negative. Three more crops of slightly less pure material were obtained by concentration of the mother liquor. Total yield 3.65 gm. (97 per cent). Several recrystallizations from methanol gave a material with a constant melting point of 116.5–117°. $[α]_D^{20} = +118°$.

Analysis—$C_{27}H_{44}O_4$. Calculated, C 74.95, H 10.25; found, C 75.26, H 10.23

3(α),12(β)-Diacetoxybisnorcholane (XI)—1 gm. of 3(α),12(β)-diacetoxy-22-bromobisnorcholane, reduced with zinc and acetic acid exactly as above, gave 0.65 gm. of oil. This was crystallized from methanol to yield long, coarse needles, m.p. 105–107°. After four recrystallizations from methanol and three from dilute ethanol, pure material melting at 115–116° was obtained. $[α]_D^{20} = +104°$.

Analysis—$C_{26}H_{42}O_4$. Calculated, C 74.60, H 10.11; found, C 74.46, H 10.36

3(α),12(β)-Diacetoxypregnane (XII)—A mixture of the two isomeric C_{20}-bromides was reduced with zinc and acetic acid in the usual manner. The total yield of crystalline product was 95 per cent. The material in the first crop melted at 156–158°. After two recrystallizations from methanol, pure crystals, m.p. 158–159.5°, $[α]_D^{20} = +112°$, were obtained.

Analysis—$C_{25}H_{40}O_4$. Calculated, 74.21, H 9.97; found, C 74.50, H 10.18

Attempted Dehydrobromination of 3(α),12(β)-Diacetoxy-23-bromonorcholane. With Collidine—Exactly 100 mg. of the 23-bromo compound were refluxed for 4½ hours with 10 cc. of freshly distilled collidine. The collidine was removed *in vacuo*, the residue taken up in ether and water, and the ethereal solution washed with water, 10 per cent sulfuric acid, water, 5 per

cent sodium carbonate solution, and finally twice with water. On removal of the ether, a red oil, weighing 88 mg., was obtained. This resisted crystallization. On chromatographic fractionation, 71 mg. of starting material were isolated. No other crystalline compound could be found.

With Piperidine—A solution of 0.22 gm. of the bromide in 5 cc. of anhydrous piperidine was heated in a sealed tube in a bath of boiling *n*-butyl alcohol (117°) for 42 hours. When the solution was cooled in an ice-bath, light, thin plates crystallized. The crystalline material did not dissolve upon addition of ether. The solvents were removed *in vacuo* and the residue taken up in ether and water. The ethereal solution was washed as described above and evaporated; an oily residue weighing 25 mg. was obtained. It may be concluded that the water-soluble piperidinium complex, stable at 117°, was formed by this treatment.

With Alcoholic Silver Nitrate—To a solution of 0.20 gm. of the 23-bromo compound in 95 per cent ethanol were added about 20 cc. of a 5 per cent solution of silver nitrate in ethanol. The solution was refluxed for 10 minutes and the precipitated silver bromide was filtered. From the solution, 20 mg. of impure crystalline material, m.p. 131–136°, were obtained by crystallization from methanol. An attempt was made to purify this by chromatographing, but only 7 mg. of material, melting at 118–121°, resulted. The nature of this compound was not determined because of lack of sufficient material.

With Sodium Ethylate—To a boiling solution of the sodium ethylate prepared from 12.4 gm. of sodium and 148 cc. of absolute ethanol was added a solution of 1.00 gm. of the 23-bromide in 52 cc. of hot absolute ethanol. Refluxing was continued for 30 minutes, and then 100 cc. of water were added. Most of the solvent was removed by distillation and the residue was poured into 1 liter of cold water. This was acidified with sulfuric acid, and the organic material removed by extraction with ether. The ethereal solution was washed with water, 5 per cent sodium carbonate solution, and again with water. The ether was removed, and the residue was dried and reacetylated in the usual manner. The resulting oil weighed 0.88 gm. This was chromatographed over alumina. The only crystalline fraction, eluted by 50 per cent benzene-50 per cent petroleum ether, yielded 50 mg. of material, m.p. 139–144°. The Beilstein test was negative. One recrystallization from methanol gave a product melting at 143–144°. $[\alpha]_D^{20} = +60°$. The analysis is satisfactory for an acetoxy ethoxy norcholene.

Analysis—$C_{27}H_{44}O_3$. Calculated, 77.83, H 10.65; found, C 77.67, H 10.85

Action of Collidine on Isomeric 3(α),12(β)-Diacetoxy-20-bromopregnane Mixture—A solution of 0.10 gm. of the 20-bromide mixture (m.p. 95–102°) in 10 cc. of collidine was refluxed for 2 hours. The solvent was removed *in*

vacuo at 80° and the residue taken up in ether and water. The aqueous washings of the ethereal solution were analyzed for halide ion by Volhard titration. They contained 1.34×10^{-5} mole of bromide, corresponding to only a 6 to 7 per cent reaction.

Action of Piperidine on Isomeric 3(α),12(β)-Diacetoxy-20-bromopregnane Mixture—Exactly 100 mg. of the isomeric 20-bromide mixture (m.p. 95–102°) were refluxed in 5 cc. of anhydrous piperidine for 22 hours. The reaction mixture was worked up in the usual manner, yielding 74 mg. of crystalline residue. This was dissolved in methanol and on cooling gave 50 mg. of crystals, melting at 162–174°. After five recrystallizations from methanol the product showed a constant melting point of 176–178.5°, $[\alpha]_D^{20} = +112°$. The analysis is correct for either 3(α),12(β)-diacetoxy-$\Delta^{17,20}$-pregnene (XIV) or 3(α),12(β)-diacetoxy-$\Delta^{20,21}$-pregnene (XV), or for a mixture of both isomers.

Analysis—$C_{25}H_{38}O_4$. Calculated, C 74.59, H 9.51; found, C 74.90, H 9.66

An additional 20 mg. of the same material were obtained by careful addition of water to the mother liquor.

SUMMARY

The diacetates of desoxycholic acid, nordesoxycholic acid, and bisnordesoxycholic acid, and the acetate of 3(α)-hydroxy-12-ketocholanic acid have been converted by the action of bromine on their silver salts to steroid bromides containing 1 less carbon atom. The bromo compounds so prepared are 3(α),12(β)-diacetoxy-23-bromonorcholane, m.p. 127.5–128.5°; 3(α),12(β)-diacetoxy-22-bromobisnorcholane, m.p. 144.5–145°; 3(α),12(β)-diacetoxy-20(α)-bromopregnane, m.p. 83–87°; 3(α),12(β)-diacetoxy-20(β)-bromopregnane, m.p. 140–142°; and 3(α)-acetoxy-12-keto-23-bromonorcholane, m.p. 209.5–211°.

These bromides have been reduced to the corresponding compounds with alkyl side chains at C_{17}: 3(α),12(β)-diacetoxynorcholane, m.p. 116.5–117°; 3(α),12(β)-diacetoxybisnorcholane, m.p. 115–116°; 3(α),12(β)-diacetoxypregnane, m.p. 158–158.5°; and 3(α)-acetoxy-12-ketonorcholane, m.p. 158.5–159.5°.

By the action of piperidine, a mixture of the isomeric diacetoxy-20-bromopregnanes has been dehydrobrominated. The product, m.p. 176–

178.5°, is a pregnenediol diacetate with the double bond at C_{17}–C_{20} or at C_{20}–C_{21}, or a mixture of both isomers.

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THE WOLFF-KISHNER REDUCTION OF 3(α),12-DIHYDROXY-11-KETOCHOLANIC ACID

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Several years ago Longwell and Wintersteiner (1) studied the properties of the 3(α),11-dihydroxy-12-ketocholanic acid, first described by Marker and Lawson (2), in the hope of accomplishing its conversion to a 3,11-dihydroxy compound by the Wolff-Kishner method. As they were unable to prepare the semicarbazone reported by these authors, they reduced the acid directly with sodium ethylate in the presence of hydrazine, and obtained an acid, $C_{24}H_{38}O_3 \cdot \frac{1}{2}H_2O$, melting at 162–163°, which was isolated via an acid succinate (m.p. 227°). Obviously both oxygen atoms in Ring C had been eliminated. Later the reaction was repeated by Marker and coworkers (3) and yielded in their hands a 3,11,12-trihydroxycholanic acid (m.p. 136°) as the sole crystallizable product. The purpose of the present reinvestigation was the thoroughgoing separation and characterization of all the products formed.

A similar study was simultaneously undertaken by Gallagher (4), in conjunction with related work which eventually led to a revision of the hitherto accepted structure of the Marker-Lawson acid as 3(α),11-dihydroxy-12-ketocholanic acid (5–8). This compound was originally prepared by hydrolysis with *hot* ethanolic potassium hydroxide solution of the amorphous 3(α),11-bromo-12-ketocholanic acid, the structure of which had been unequivocally proved (1), but was recently shown by Seebeck and Reichstein (9) and independently by Gallagher and Long (7) to consist of a mixture of 11-epimers. Gallagher and his collaborators (7, 8) found that either epimer is converted into the Marker-Lawson acid by the above treatment, but that when the hydrolysis is conducted with *cold* aqueous alcoholic sodium hydroxide solution, the two epimeric 11-bromo acids give rise to two different dihydroxyketocholanic acids, neither of which is identical with the Marker-Lawson acid. In contrast to the latter these new isomers readily form hydrazones. It is therefore highly probable that the two new acids are 12-keto acids which differ from each other by epimerism at C_{11}. The Marker-Lawson acid consequently must be a 3(α),12-dihydroxy-11-ketocholanic acid, which at once explains the inertness of its keto group. These results were communicated to us by the Chicago investigators in the continuous exchange of information which accompanied the progress of

these studies in both laboratories.　We take great pleasure in acknowledging the whole hearted cooperation we have enjoyed from the Chicago group in this problem.

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TABLE I

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Wolff-Kishner Reduction of $3(\alpha),12(\alpha\ ?)$-Dihydroxy-11-ketocholanic Acid

I to III, starting acid and derivatives; IV to XV, reduction products and derivatives.

	M.p.	$[\alpha]_D$	
	°C.	*degrees*	
I. $3(\alpha),12(\alpha\ ?)$-Dihydroxy-11-ketocholanic acid.	205	+67	(Ethanol)
II. Methyl $3(\alpha),12(\alpha\ ?)$-dihydroxy-11-ketocholanate..................................	157	+64.6	"
III. Methyl $3(\alpha),12(\alpha\ ?)$-diacetoxy-11-ketocholanate..................................	69	+54.5	"
IV. Methyl $3(\alpha)$-acetoxy-Δ^{14}-cholenate..........	119	+50.2	(Acetone)
V. $3(\alpha)$-Hydroxy-Δ^{11}-cholenic acid.............	165	+33.2	(Ethanol)
VI. Methyl $3(\alpha),12(\alpha\ ?)$-diacetoxy-11(β)-hydroxycholanate...........................	212	+59.9	(Chloroform)
VII. $3(\alpha),11(\beta),12(\alpha\ ?)$-Trihydroxycholanic acid...................................	177	+42.9	(Ethanol)
VIII. Methyl $3(\alpha),11(\alpha)$-diacetoxy-12(β)-hydroxycholanate...........................	129	+39.6	(Chloroform)
IX. Methyl $3(\alpha),11(\alpha),12(\beta)$-triacetoxycholanate..	187	+64.5	"
X. $3(\alpha),11(\alpha),12(\beta)$-Trihydroxycholanic acid....	164	+46.9	(Ethanol)
XI. Methyl $3(\alpha),11(\alpha),12(\alpha)$-triacetoxycholanate	164	+37.1	(Chloroform)
XII. $3(\alpha),11(\alpha),12(\alpha)$-Trihydroxycholanic acid.	174	+26.8	(Ethanol)
XIII. Methyl $3(\alpha),11(\alpha),12(\alpha)$-trihydroxycholanate..	135	+28.4	"
XIV. Methyl $3(\alpha),11(\alpha)$-diacetoxy-12-ketocholanate..	151	+34.9	(Ethanol)
XV. $3(\alpha),11(\alpha)$-Dihydroxy-12-ketocholanic acid..	193	+84.9	"

<div align="center">

Results

</div>

The essential findings are summarized in Table I.　The use, in some experiments, of the methyl ester II or of the diacetyl methyl ester III, instead of the acid I, as the starting material did not modify the course of the reaction.　It should be mentioned here that the ester III, methyl $3(\alpha),12$-diacetoxy-11-ketocholanate, which had been described previously as an

amorphous product (1), has now been obtained in crystalline form. The inertness of the keto group in the acetylated ester was just as marked as in the free acid. In the attempted preparation of the oxime and semicarbazone, the ester was recovered unchanged, while treatment with hydrazine resulted merely in the formation of the hydrazide of an acid in which one of the acetyl groups, presumably at C_3, had been lost by saponification.

It was found that the separation of the complex mixture of reduced acids could be best accomplished after their conversion into acetylated methyl esters. The acetylation was carried out at room temperature, which left hydroxyl groups relatively resistant to esterification in the free state and thus facilitated the separation and differentiation of the stereoisomers present. One of the partially acetylated esters (VI) could be secured by direct crystallization; the other three reaction products were obtained by chromatographic fractionation of the remaining mixture. This procedure was applied to three batches of reduced material and proved to be well reproducible, although the yields of the four compounds were small and somewhat variable.

The isolated compounds were (1) an ester, $C_{27}H_{42}O_4$, identified by melting point and rotation as the methyl $3(\alpha)$-acetoxy-Δ^{11}-cholenate (IV) which has been recently prepared by Press and Reichstein (10) and by Kendall and his collaborators (11) through other routes. The corresponding free acid (V) melted at 165°, as reported by the Swiss authors. The preparation of the acid succinate, m.p. 227°, confirmed the identity of the present reaction product with the acid, m.p. 163°, described in our earlier paper (1); (2) an ester, m.p. 212°, which had the composition of a methyl diacetoxyhydroxycholanate ($C_{29}H_{46}O_7$). Since on oxidation with chromic acid this compound yielded the methyl $3(\alpha)$,12-diacetoxy-11-ketocholanate III, it must be a methyl $3(\alpha)$,12-diacetoxy-11-hydroxycholanate (VI). The ester was hydrolyzed by hot alkali to a $3(\alpha)$,11,12-trihydroxycholanic acid (VII) melting at 177°. That no configuration change had occurred in the hydrolysis was proved by conversion of the acid VII into an amorphous methyl ester which on acetylation at room temperature quantitatively reformed the original diacetoxy hydroxy ester VI. However, an attempt to force the acetylation of the free hydroxyl group in the latter by conducting the reaction at elevated temperature resulted in the formation of pigmented decomposition products; (3) a methyl diacetoxyhydroxycholanate, m.p. 129° (VIII), which differs from the isomer VI in that on oxidation with chromic acid it yielded a new methyl diacetoxyketocholanate (XIV) melting at 151°. Furthermore, unlike VI, the ester VIII is amenable to vigorous acetylation, which in this case afforded a methyl 3,11,12-triacetoxycholanate (IX) melting at 187°. The hydrolysis product of VII was a trihydroxycholanic acid (X) differing from the isomeric acid VII by its lower melting point (164°) and its slightly higher dextrorotation.

The diacetoxy keto ester XIV on hydrolysis with 0.5 N methanolic potassium hydroxide at room temperature yielded nearly quantitatively a dihydroxyketocholanic acid (XV) melting at 193°, which differs from the starting acid I by its higher dextrorotation (+84.9° as against +67° for I). However, when the hydrolysis was conducted with a stronger alkali at boiling temperature, a mixture was obtained from which only small amounts of dihydroxyketocholanic acid melting at 201–203° could be secured by repeated recrystallization. The melting point of a mixture with the starting acid I was not depressed, but the rotation (+72.7°) indicated that the hydrolysis product, I, was contaminated with some of the more dextrorotatory isomer XV.

The reason for this behavior became obvious when Gallagher and Hollander (8) discovered that hydrolysis of methyl 3(α)-hydroxy-11(β)-bromo-12-ketocholanate with cold aqueous alkali yielded a 3(α),11-hydroxy-12-ketocholanic acid identical with XV. Treatment of this acid with hot alkali, as mentioned before, causes rearrangement to the 11-keto acid I. Consequently, the free hydroxyl group in the diacetoxy hydroxy ester VIII, from which XIV was obtained, must be in position 12. It furthermore follows from the resistance of this group to acetylation at room temperature that it is more strongly hindered than the 11-hydroxyl group; but this inertness is only relative and can be overcome by more energetic acetylation; (4) a methyl 3,11,12-triacetoxycholanate (XI) melting at 163°[1] differing from the isomeric compound IX by its considerably lower dextrorotation. Hydrolysis of XI with 0.5 N methanolic potassium hydroxide at room temperature readily afforded a 3,11,12-trihydroxycholanic acid melting at 173–174° (XII), which markedly depressed the melting point of the isomeric acid, m.p. 177° (VII). The trihydroxy acid XII was further characterized by the preparation of a crystalline methyl ester, m.p. 134–135.5° (XIII). However, reacetylation of this methyl ester at room temperature yielded a mixture from which the original triacetoxy ester XI could be secured only by chromatographing. This may be taken as evidence that one of the hydroxyl groups is hindered to a certain extent, but certainly less strongly than in acids VII and X.

Acid XII seems to occur in another polymorphous modification, or else is not quite stable, since recrystallized preparations invariably showed some sintering at about 135° before melting at the temperature indicated. This observation is mentioned here because it is the only indication in our work for the presence of the 3,11,12-trihydroxycholanic acid, m.p. 136°, described by Marker and his colleagues (3). The yield obtained by these authors was 27 per cent. It is highly improbable that their acid is a fourth isomer not encountered by us, as it could hardly have escaped isolation in

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[1] The melting point of 164° given in Table I refers to that of a purer specimen obtained by the route V → XII → XI.

the chromatographic fractionation employed in our work. It may be a polymorphous modification of our acid XII, or, more likely, an isomeric mixture. Since Marker's acid has not been characterized by rotation data or derivatives, a satisfactory correlation with our compounds is not possible.

It seemed of interest to ascertain which, if any, of the isolated trihydroxycholanic acids could be obtained by addition of hydroxyl groups to the 11–12 double bond in $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid (V) by means of osmium tetroxide. This reagent, which is known to give rise to *cis*-glycols (12), has been applied to the desoxy analogue of V, Δ^{11}-cholenic acid, by Alther and Reichstein (13) and yielded an 11,12-dihydroxycholanic acid. In our case the reaction led to the trihydroxy acid XII, which therefore must be one of the two possible 11,12-*cis* isomers. The yield of XII indicated that this isomer is the principal, if not the sole, product.

DISCUSSION

It is clear from the foregoing that the reduction of $3(\alpha)$,12-dihydroxy-11-ketocholanic acid (I) with sodium ethylate and hydrazine at 200° leads primarily to stereoisomeric 3,11,12-trihydroxycholanic acids. The reduction of the 11-keto group to carbinol is reminiscent of the "abnormal" Wolff-Kishner reduction of the semicarbazones or hydrazones of 3-keto-steroids to 3-hydroxy compounds (14), but this similarity is superficial in so far as in the latter cases the addition of hydrazine forces the reaction to proceed normally; that is, with the formation of a methylene group. With the present substrate, the cause of the "abnormal" reduction is probably to be sought in the demonstrated inability of the keto group to react with hydrazine or its derivatives, so that the reduction of this group by sodium ethylate, which is known under these conditions to yield alcohols from 3-ketones (14), becomes the prevailing reaction. This concept is supported by the fact, demonstrated in the following paper, that in $3(\alpha)$-hydroxy-11,12-diketocholanic acid the 12-keto group is reduced in the normal manner, that is with elimination of the carbonyl oxygen, while the 11-keto group is transformed into a hydroxyl group. With the present starting product this reaction, *per se*, would account for the formation of two trihydroxy acids differing from each other by epimerism at C_{11}. The formation of the third trihydroxy acid isolated by us, and of a fourth isomer encountered in small amounts by Gallagher (4) instead of our acid VII, calls for an additional mechanism entailing partial epimerization of the original 12-hydroxyl group. Most likely this epimerization occurs *before* the reduction through enolization of the 11-keto group to an 11,12-enediol. Since the enediol form is common to the epimeric 12-hydroxy-11-keto acids and the epimeric 11-hydroxy-12-keto acids, all four of these acids could very well coexist in hot alkaline solution and function as potential precursors of

the stereoisomeric trihydroxy acids. The findings of Gallagher and his colleagues that both epimeric $3(\alpha)$,11-dihydroxy-12-ketocholanic acids are readily isomerized by hot alkali to the Marker-Lawson acid do not invalidate this assumption, as the extent of this conversion is not known. To judge from the yields, the isomerization is by no means quantitative, and furthermore the isolation of the Marker-Lawson acid is favored by the relatively greater ease with which it can be separated in crystalline form. Indeed, a reasonable argument for the presence of at least one of the 11-hydroxy-12-keto acids before the start of the reduction can be made on the following grounds.

It is difficult to visualize the formation of $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid from the Marker-Lawson acid by a mechanism other than elimination of the keto group, and subsequent dehydration of the remaining hydroxyl group in Ring C to form the 11–12 double bond. The available evidence indicates that an 11-keto group resists the usual procedures for reduction to a methylene group, but that a 12-keto group can be so reduced by sodium ethylate and hydrazine. Consequently, the immediate precursor of the unsaturated acid must be a $3(\alpha)$,11-dihydroxycholanic acid which in turn arises by normal reduction from a $3(\alpha)$,11-dihydroxy-12-ketocholanic acid formed by partial isomerization of the starting material. The fact that none of the intermediate 3,11-dihydroxy acid was found among the reduction products may be merely due to the inadequacy of the method of separation, or may be ascribed, as by Long and Gallagher (6), to the presence in this acid of an $11(\beta)$-hydroxyl group which these authors assume to be susceptible to dehydration under these conditions.

It is possible to draw from our results, in conjunction with those of Gallagher and his colleagues, certain conclusions regarding the configurations of the 11- and 12-hydroxyl groups in the stereoisomeric trihydroxy-cholanic acids VII, X, and XII. Stereoisomerism at C_3, such as might have resulted from an inversion of the $3(\alpha)$-hydroxyl group under the conditions of the Wolff-Kishner reaction (14), is excluded by the fact that the three acids, as well as the fourth isomer of Gallagher (4), have been linked with $3(\alpha)$-hydroxyl compounds by reactions which could not cause inversion at this carbon atom (VII → I; X → XV; V → XII).

In regard to the "absolute" configurations of 11-hydroxy compounds, the following facts must be considered. The space model shows that an 11-hydroxyl group is more strongly hindered in the β (*cis* to the methyl groups at C_{10} and C_{13}) than in the α configuration. Methyl 11-hydroxy-cholanate (15), and methyl $3(\alpha)$-acetoxy-11-hydroxycholanate (16) require rather severe conditions for acetylation at C_{11}, and in this respect resemble the adrenal cortical steroids. Reich and Reichstein (15) tentatively assigned the $11(\alpha)$ configuration to methyl 11-hydroxycholanate, because it

was one of the products formed in the catalytic reduction of an 11,12-oxide ester which they showed to be methyl $11(\alpha),12(\alpha)$-oxidocholanate. They admit, however, the possibility of a Walden inversion in the reaction, and consequently the limited value of this type of evidence. On the other hand, our experience with the triacetoxy ester XI and the 3,11-diacetoxy-12-hydroxy ester VIII proves conclusively that a hydroxyl group in position 11 can exist in a spatial orientation in which it is easily accessible to acetylating agents. Another case in point is the $3(\alpha)$,11-dihydroxycholanic acid which Gallagher and his associates (6, 8) obtained by Wolff-Kishner reduction of both 11-epimeric $3(\alpha)$,11-dihydroxy-12-ketocholanic acids, and which yields a diacetate on relatively mild acetylation. It is therefore logical to accord the $11(\alpha)$ configuration only to 11-hydroxy compounds which show this behavior, and the β configuration to those which do not.

In the attempt to assign configurations to the 12-hydroxyl groups in the reduced acids, the epimeric pair desoxycholic acid and 12-epidesoxycholic acid must serve for reference. Koechlin and Reichstein (17) adduced evidence by saponification rate measurements that the 12-hydroxyl group in desoxycholic acid is more strongly hindered than in its epimer. We have found that desoxycholic acid under the conditions of acetylation used by us yielded the 3-monoacetate as the only crystallizable product (m.p. 124–126°; yield about 40 per cent). This, in conjunction with the fact that the 12-hydroxyl group in I and in VII is quantitatively acetylated under these conditions, provides additional evidence for the (moderately) hindered character of this group in desoxycholic acid and the absence of such hindrance in the epimeric configuration. Since the difference in the reactivity of the epimeric groups is probably not great, this criterion for correlating configurations must admittedly be used with caution. However, a check is provided by the rotation data. Desoxycholic acid and its derivatives have higher dextrorotations than their 12-epimers. Therefore, if it can be shown that two compounds differ by epimerism at C_{12} only, the epimer giving evidence of being more hindered should also be more dextrorotatory than the other. If both these criteria are met, this epimer may be assumed to be configurationally related to desoxycholic acid.

In regard to the "absolute" configuration of the 12-hydroxyl group in desoxycholic acid and its 12-epimer opinions are divided. Various theoretical considerations led Koechlin and Reichstein (17) to assign the $12(\beta)$ configuration (12-hydroxyl group cis to methyl at C_{13}) to desoxycholic acid and the $12(\alpha)$ configuration to the epi compound. Gallagher and Long (5) present evidence which favors the opposite relationship. However, as will be shown below, the latter view when applied to the epimeric trihydroxycholanic acids conflicts with the data concerning relative hindrance and rotation, *provided that the absolute configurations at C_{11} in these compounds*

have been correctly assigned. We have therefore adopted the view of the Swiss investigators as the basis of the stereochemical relationship discussed here, but wish to make it clear that the designations thus arrived at should be considered as tentative. They may have to be revised as more facts pertaining to the absolute configurations at C_{11} and C_{12} become available.

On the premises set forth, acid XII has to be designated $3(\alpha),11(\alpha),-12(\alpha)$, because none of the hydroxyl groups is markedly hindered, and the *cis* relationship of the 11- and 12-hydroxyl groups has been established by its formation from $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid with osmium tetroxide. Acid X possesses a moderately hindered hydroxyl group, which has been shown to occupy the 12 position, since the diacetoxy hydroxy methyl ester VIII can be oxidized to the methyl $3(\alpha),12(\alpha)$-diacetoxy-12-ketocholanate of Gallagher and Hollander (8). Consequently X is $3(\alpha),11(\alpha),12(\beta)$-trihydroxycholanic acid, the 12-epimer of XII. The configurations assigned to XII and X are in agreement with those proposed by Gallagher (4). However, the facts regarding steric hindrance as well as the rotations (X more dextrorotatory than XII) relate X configurationally to desoxycholic acid and XII to 12-epidesoxycholic acid. This would seem to contradict the view of Gallagher and Long that desoxycholic acid is a $12(\alpha)$ compound, unless it is assumed that the presence of an $11(\alpha)$-hydroxyl group in X and XII reverses the relative reactivities of the 12-hydroxyl group as well as their contributions to rotation.

Acid VII has a strongly hindered hydroxyl group occupying position 11, as shown by the oxidation of its diacetoxy hydroxy methyl ester VI to the diacetoxy ester III of the starting acid. Since the other two hydroxyl groups can be quantitatively acetylated at room temperature, it must be assumed by analogy with the epimeric pair XII and X that the 12-hydroxyl group has the α configuration. Acid VII is therefore designated $3(\alpha),-11(\beta),12(\alpha)$-trihydroxycholanic acid, and the starting acid I, $3(\alpha),-12(\alpha)$-dihydroxy-11-ketocholanic acid. This is contrary to the designation of Gallagher (4), who assigns to these acids the $12(\beta)$ configuration and considers the fourth isomeric trihydroxycholanic acid isolated by him to be the $12(\alpha)$-epimer. Reacetylation has been carried out by this author on the corresponding methyl $3(\alpha),12$-dihydroxy-11-acetoxycholanate obtained by acetolysis of $3(\alpha)$-hydroxy-11,12-oxidocholanic acid and subsequent esterification. A triacetate was obtained, but since the reaction was conducted at elevated temperature, the reactivity of the 12-hydroxyl group in this isomer relative to that in acid VII cannot be judged. The specific rotations in ethanol of VII ($+42.9°$) and of the isomer of Gallagher ($+54°$) indicate that the former is related to 12-epidesoxycholic acid and the latter to desoxycholic acid. This is of course compatible with Gallagher's viewpoint ($12(\alpha)$ configuration in desoxycholic acid and in his fourth isomeric

trihydroxycholanic acid, 12(β) configuration in 12-epidesoxycholic acid and in VII), as well as with our own, which reverses the designations in both these pairs. We prefer the latter view, because otherwise it will have to be assumed that the postulated optical relationships with the desoxycholic acid pair hold for the pair VII and the isomer of Gallagher, but not for the pair XII and X. However, since the evidence for either configuration in acid VII is in our opinion rather tenuous, a question mark following the 12(α) designation adopted by us for VII and I and their derivatives is in order.

EXPERIMENTAL

The starting material was prepared by the method previously described (1), except that we found it more convenient to use for the bromination in position 11 the methyl ester of 3(α)-acetoxy-12-ketocholanic acid instead of the acid itself. In the preparation of a later batch, in which the method of Gallagher and Long (7) was followed, we made the observation that hydrolysis of the crystalline 3(α)-acetoxy-11(α)-bromo-12-ketocholanic acid methyl ester with methanolic potassium hydroxide solution at room temperature did not lead to replacement of the bromine atom; so that the resulting product was the hitherto undescribed 3(α)-hydroxy-11(α)-bromo-12-ketocholanic acid. Subsequent treatment with sodium hydroxide in aqueous ethanol at room temperature yielded the insoluble sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid, which was then isomerized to the Marker-Lawson acid with hot aqueous alkali.

In connection with the stereochemical questions previously discussed, it seemed of interest to examine the behavior of methyl 3(α),11(β)-dihydroxy-12-ketocholanate on acetylation at room temperature. As expected, a monoacetate, methyl 3(α)-acetoxy-11(β)-hydroxy-12-ketocholanate (m.p. 161°), was obtained.

3(α)-Hydroxy-11(α)-bromo-12-ketocholanic Acid—Methyl 3(α)-acetoxy-12-ketocholanate was prepared by the method of Reichstein and Sorkin (18) and brominated as previously described for the corresponding 3(α)-acetoxy keto acid (1), except that a small amount of hydrobromic acid was added and the temperature was maintained at 65–70° instead of at 55°. For routine purposes, the procedure previously given (1) was then followed, but in later experiments the crude bromination product was reesterified with diazomethane in ether solution. On evaporation of the ether, most of the material crystallized. Recrystallization from the same solvent yielded large needles, which by melting point (159–161°) and rotation ($[\alpha]_D^{24} = +49.1°$ in chloroform) were identified as methyl 3(α)-acetoxy-11(α)-bromo-12-ketocholanate (7, 9). A solution of 10 gm. of the ester in 125 cc. of methanolic 0.5 N potassium hydroxide solution was allowed to stand at

room temperature for 2 days and then separated into acidic and neutral fractions. The saponified part (4.0 gm.) crystallized from ether in needles melting at 194–196°. After recrystallization from 80 per cent alcohol, the melting point was 190–191.5° (gas evolution). More of the crude acid could be recovered by subjecting the neutral fraction to a more prolonged hydrolysis under the same conditions; $[\alpha]_D^{22} = +44.5°$ (1.04 per cent in alcohol).

Analysis—$C_{24}H_{37}O_4Br$. Calculated. C 61.38, H 7.95, Br 17.03
Found. " 61.43, " 7.88, " 16.59

Methyl 3(α)-Acetoxy-11(β)-hydroxy-12-ketocholanate—6.0 gm. of 3(α)-hydroxy-11(α)-bromo-12-ketocholanic acid were converted into the insoluble sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid with cold aqueous alcoholic sodium hydroxide solution according to the directions of Long and Gallagher (6) for the acetoxy methyl ester. 600 mg. of the salt (m.p. 200–203°) were suspended in 15 cc. of alcohol and 200 cc. of water. After the addition of 2 cc. of 2 N sulfuric acid the mixture was shaken mechanically for 2 hours, and then extracted with ether. The residue of the dried and evaporated ether solution (590 mg.) was esterified with diazomethane in ether, and the crude ester was acetylated with 5 cc. of pyridine and 2.5 cc. of acetic anhydride at room temperature. After 24 hours, the mixture was worked up in the usual way by extraction with ether. The ether residue was recrystallized repeatedly from ether-pentane and finally from methanol, from which it formed plates melting at 159–161°; $[\alpha]_D^{22} = +118.4°$ (0.88 per cent in alcohol).

Analysis—$C_{27}H_{42}O_6$. Calculated, C 70.08, H 9.16; found, C 69.90, H 9.02

The sodium salt of 3(α),11(β)-dihydroxy-12-ketocholanic acid was isomerized by boiling in 5 per cent aqueous sodium hydroxide solution, and acid I was obtained in good yield.

Methyl 3(α),12(α ?)-Diacetoxy-11-ketocholanate (III)—A solution of 500 mg. of methyl 3(α),12(α ?)-dihydroxy-11-ketocholanate in 2 cc. of pyridine and 1 cc. of acetic anhydride was allowed to stand at room temperature for 24 hours. The reaction product, isolated by ether extraction, crystallized after addition of a little methanol. It was recrystallized repeatedly from about 3 parts of the same solvent, from which it formed fine long needles on standing in the refrigerator. The melting point of the purified product was 67–69°, but occasionally higher values (up to 80°) were observed; $[\alpha]_D = +54.5°$ (0.98 per cent in alcohol).

Analysis—$C_{29}H_{44}O_7$. Calculated, C 69.02, H 8.83; found, C 68.76, H 8.69

A solution of 45.2 mg. (0.0896 mM) in 0.1 N methanolic potassium hydroxide solution was allowed to stand 6 days at room temperature. Back titration showed that 0.260 milliequivalent of base had been neutralized (calculated, 0.269 milliequivalent).

3(α)-Hydroxy-12(α ?)-acetoxy-11-ketocholanic Acid Hydrazide—A solution of the above ester (208 mg.) in absolute ethanol (5 cc.) and 85 per cent hydrazine hydrate (0.5 cc.) was boiled under a reflux for 20 hours. The oil remaining after evaporation of the alcohol was dissolved in 1 cc. of methanol. After addition of a few drops of water and standing in the refrigerator a crystalline product separated. This was recrystallized twice from 20 per cent aqueous methanol, yielding rosettes of needles (45 mg.) which melted at 123–124°.

Analysis—$C_{26}H_{42}O_5N_2$. Calculated. C 67.48, H 9.16, N 6.06
Found. " 67.52, " 9.35, " 6.58

Wolff-Kishner Reduction of 3(α),12(α ?)-Dihydroxy-11-ketocholanic Acid (I)—2 to 3 gm. batches of the acid, or of its derivatives II or III, were heated together with sodium ethylate, prepared from an equal amount of sodium and 10 parts of absolute ethanol, and 2.5 cc. of 85 per cent hydrazine hydrate in a sealed tube at 190–200° for 6 hours. The contents were poured into water, and the acidified solution was extracted with ether. The dried ether solution was evaporated to a small volume and treated with an excess of diazomethane. After several hours, it was taken to dryness and the residue was allowed to stand with pyridine-acetic anhydride, 2:1, at room temperature for 24 hours. The subsequent treatment of the acetylated ester mixture is illustrated by a typical experiment.

The resinous product (about 4 gm.) was dissolved in about 10 cc. of absolute methanol. After standing for some time in the refrigerator the solution deposited a mass of fine needles, which were filtered and washed with cold methanol (Fraction A, 320 mg.). The mother liquor was brought to dryness, and the residue was dissolved in 100 cc. of benzene-hexane, 4:1, and adsorbed on a column of aluminum oxide (3.5 × 22 cm.). The chromatogram was developed with 500 cc. of the same solvent mixture, and elution was effected by washing with benzene-hexane, 1:1 (1800 cc.), benzene (2000 cc.), and ether-benzene, 1:4 (600 cc.). The filtrates were collected in 100 cc. portions. Most of the benzene-hexane, 1:1, eluates crystallized spontaneously and were combined (Fraction B, 250 mg., m.p. 114–116°). The first benzene eluates were also for the most part crystalline (Fraction C, 708 mg., m.p. 145–149°). The amorphous residues of the remaining benzene eluates were dissolved separately in amounts of ether-hexane, 1:4, just sufficient to effect solution. After several days· in

the refrigerator, some of the solutions deposited crystalline material, which was combined (Fraction D, 303 mg., m.p. 75–90°). The ether-benzene eluates could not be crystallized. They were combined (Fraction E, 1.5 gm.) and oxidized with chromic acid, whereby they yielded appreciable amounts of the diacetoxy keto ester XIV.

The separation of the various constituents of the ester mixture by the fractional elution procedure described above is not as efficient as might be desired. There is only a gradual decrease in the weight of the residues from the 100 cc. portions as elution with the same solvent proceeds, and consequently crystallizability and melting points rather than weight trends must be relied upon as criteria for combining these eluates into the several fractions mentioned.

Methyl 3(α)-Acetoxy-Δ¹¹-cholenate (IV)—Fraction B was recrystallized twice from methanol, from which it formed large elongated platelets (196 mg.) melting at 118–119°; $[α]_D^{23} = +50.2°$ (0.85 per cent in acetone); $+45.7°$ (0.87 per cent in chloroform). Press and Reichstein (10) report a melting point of 116–117° and an $[α]_D^{14}$ of $+52.2°$ in acetone.

Analysis—$C_{27}H_{42}O_4$. Calculated, C 75.29, H 9.84; found, C 75.56, H 9.89

In one case, the melting point of Fraction B was 117–121°, and that of the purified product 121–122°. Although the latter showed the same characteristic crystal form as the lower melting preparations previously obtained, it is possible that in this material some of the isomeric ester, methyl 3(α)-acetoxy-Δ⁹,¹¹-cholenate (m.p. 138°), or of the completely reduced ester, methyl 3(α)-acetoxycholanate (m.p. 134°), was present as an impurity. Seebeck and Reichstein (9) have shown that the three acetylated esters form inseparable mixtures with melting points intermediate between those of the components.

3(α)-Hydroxy-Δ¹¹-cholenic Acid (V)—Hydrolysis of 140 mg. of ester IV, effected by boiling with 5 per cent methanolic potassium hydroxide solution for 30 minutes, yielded prisms which after recrystallization from acetone melted at 163–165°; $[α]_D^{22} = +33.2°$ (1.32 per cent in ethanol). Press and Reichstein (10) report a melting point of 165–166° and an $[α]_D^{11}$ of $+33.2°$ in ethanol. A part of this material (71 mg.) was converted into the acid succinate with pyridine and succinic anhydride. The resulting product, recrystallized from dilute alcohol, melted at 226–227°, as did the derivative previously obtained (1).

Methyl 3(α),12(α ?)-Diacetoxy-11(β)-hydroxycholanate (VI)—Fraction A (320 mg., m.p. about 190°) was recrystallized four times from methanol, whereby 100 mg. of small rods melting at 210–212° were obtained; $[α]_D^{25} = +59.9°$ (0.89 per cent in chloroform).

Analysis—$C_{29}H_{44}O_7$. Calculated, C 68.73, H 9.15; found, C 68.85, H 9.27

Oxidation of 42 mg. of ester VI with 25 mg. of chromium trioxide in 1 cc. of pure acetic acid at room temperature for 24 hours yielded in the neutral fraction 41 mg. of a glass, which, when moistened with methanol, turned into a mass of long, fine needles on standing in the refrigerator. The substance (m.p. 68–70°) was recrystallized twice from small volumes of methanol and then melted at 67–69°. The melting point was not depressed by admixture of methyl $3(\alpha),12(\alpha\ ?)$-diacetoxy-11-ketocholanate (III).

$3(\alpha),11(\beta),12(\alpha\ ?)$-Trihydroxycholanic Acid (VII)—The ester VI (135 mg.) was hydrolyzed by boiling with 5 per cent methanolic potassium hydroxide solution for 1 hour. The product was recrystallized twice from ethyl acetate, yielding rhombohedral crystals melting at 176–177°; $[\alpha]_D^{24} = +42.9°$ (0.97 per cent in ethanol).

Analysis—$C_{24}H_{40}O_5$. Calculated, C 70.53, H 9.87; found, C 70.57, H 9.97

Treatment of the trihydroxy acid with diazomethane resulted in the formation of a colorless resin which could not be crystallized. The amorphous ester (21 mg.) was acetylated with acetic anhydride and pyridine at room temperature. The recrystallized product (17.9 mg.) melted at 211–213°. A mixture with ester VI showed the same melting point.

Methyl $3(\alpha),11(\alpha)$-Diacetoxy-12(β)-hydroxycholanate (VIII)—Fraction D (303 mg.) on recrystallization from ether-pentane, 1:2, yielded 128 mg. of long needles melting at 123° after softening at 97°. After three more recrystallizations from the same solvents, the melting point became constant at 127–129°; $[\alpha]_D^{25} = +39.6°$ (0.91 per cent in chloroform).

Analysis—$C_{29}H_{46}O_7$. Calculated, C 68.73, H 9.15; found, C 68.86, H 9.11

Methyl $3(\alpha),11(\alpha),12(\beta)$-Triacetoxycholanate (IX)—The ester VIII (38 mg.) was boiled with 2 cc. of pyridine and 1 cc. of acetic anhydride for 5 hours. The partly crystalline reaction product was adsorbed from a benzene-hexane solution, 1:9, on a small column of aluminum oxide. Benzene-hexane, 1:1, eluted homogeneous looking material (10 mg.) which, after recrystallization from methanol, melted at 186–187°; $[\alpha]_D^{23} = +64.5°$ (0.31 per cent in chloroform).

Analysis—$C_{31}H_{48}O_8$. Calculated, C 67.84, H 8.82; found, C 67.87, H 8.83

$3(\alpha),11(\alpha),12(\beta)$-Trihydroxycholanic Acid (X)—The ester VIII (52 mg.) was hydrolyzed by boiling with 5 per cent methanolic potassium hydroxide solution for 3 hours. The acid was recrystallized from ethyl acetate, from which it formed blunt rods melting at 162–164°; $[\alpha]_D^{23} = 46.9°$ (0.78 per cent in ethanol).

Analysis—$C_{24}H_{40}O_5$. Calculated, C 70.53, H 9.87; found, C 70.61, H 10.11

Methyl 3(α),11(α),12(α)-Triacetoxycholanate (XI)—Fraction C was recrystallized twice from methanol and then twice from hexane. The compound crystallized from either solvent in long, fine needles. The melting point of the pure compound (159 mg.) was 161.5–163°; $[\alpha]_D^{23}$ = +37.1° (1.05 per cent in chloroform).

Analysis—$C_{31}H_{48}O_8$. Calculated, C 67.84, H 8.82; found, C 67.94, H 8.81

3(α),11(α),12(α)-Trihydroxycholanic Acid (XII)—Slightly impure ester XI (177 mg., m.p. 158–162°) was dissolved in 5 cc. of 5 per cent methanolic potassium hydroxide solution, and the mixture allowed to stand at room temperature for 68 hours. The reaction product was dissolved in a small volume of warm ethyl acetate. After standing overnight in the refrigerator, clear cut hexagonal plates mixed with a small amount of blunt needles had formed. The latter went into solution again at room temperature. The plates were filtered and washed well with cold ethyl acetate. This material (79 mg.) melted sharply at 173–174°. On recrystallization from the same solvent, small rods were obtained which underwent marked shrinking at 136°, thereby forming an opaque mass which collapsed and became clear at 173°. Further recrystallization did not change this behavior. A mixture with the isomeric acid VII (m.p. 176–177°) melted at 134–148°; $[\alpha]_D^{23}$ = +26.7° (0.96 per cent in ethanol).

Analysis—$C_{24}H_{40}O_5$. Calculated, C 70.53, H 9.87; found, C 70.54, H 9.71

When 123 mg. of pure ester XI were hydrolyzed by boiling with 5 per cent methanolic potassium hydroxide solution for 3 hours, the resulting rods, recrystallized once from ethyl acetate, showed unsatisfactory melting properties (sintering at 134°, clear at 173°) which did not materially improve on further recrystallization. The rotation of the final preparation (38 mg.) was +17.9° in alcohol.

Methyl 3(α),11(α),12(α)-Trihydroxycholanate (XIII)—A solution of acid XII (38 mg.) in ether was esterified with diazomethane and yielded, on evaporation of the solvent, rosettes of large square plates melting at 65–74°. On recrystallization from ether-pentane, the crystal form changed to needles. These melted at 133–136° and probably represent another polymorphous modification. The melting point of the pure compound was 134–135.5°; $[\alpha]_D^{23}$ = +28.4° (0.875 per cent in ethanol).

Analysis—$C_{25}H_{42}O_5$. Calculated, C 71.03, H 10.02; found, C 70.90, H 9.86

3(α),11(α),12(α)-Trihydroxycholanic Acid (XII) from 3(α)-Hydroxy-Δ¹¹-cholenic Acid (V)—3(α)-Hydroxy-Δ¹¹-cholenic acid[2] was converted into

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[2] We wish to thank Dr. Randolph T. Major of Merck and Company, Inc., for the gift of several gm. of the pure compound.

the acetoxy ester IV. A solution of 1.5 gm. of the ester and 1.0 gm. of osmium tetroxide in 100 cc. of dry ether was allowed to stand for 6 days. The solvent was removed, and the black residue was taken up in a mixture of 10 cc. of benzene and 50 cc. of ethanol. After the addition of a solution of 2 gm. each of potassium hydroxide and sodium sulfite in 12 cc. of water, the mixture was boiled for 3 hours. A filtered solution of 2 cc. of saturated aqueous sodium chloride solution and 8 cc. of ethanol was added. After the removal of the benzene by distillation, the brown precipitate was filtered from the hot solution and washed well with hot ethanol saturated with sodium chloride. The filtrate was neutralized and after removal of most of the alcohol *in vacuo* and acidification with hydrochloric acid was extracted with ether. On concentration of the dried ether solution crystalline material (522 mg.) separated, which after recrystallization from ethyl acetate melted at 172–173°. The melting point of a mixture with acid XII was not depressed. $[\alpha]_D^{22}$ in ethanol (+20.6°) was somewhat lower than that of the reference sample.

Esterification with diazomethane yielded the methyl ester XIII melting at 135–135.5°. When 147 mg. of the latter were acetylated in the usual manner at room temperature, the resulting product was inhomogeneous (m.p. 70–128°). It was purified by chromatographing and yielded 63 mg. of the triacetoxy ester XI, melting at 163–164°; $[\alpha]_D = +33.9°$ (0.71 per cent in chloroform).

The material from the original mother liquor was esterified and acetylated, and on chromatographing yielded 258 mg. more of the pure ester XI.

Methyl 3(α),11(α)-Diacetoxy-12-ketocholanate (XIV)—A solution of 38.4 mg. of methyl 3(α),11(α)-diacetoxy-12(β)-hydroxycholanate (VIII) and 16 mg. of chromium trioxide in 1 cc. of pure acetic acid was allowed to stand for 24 hours at room temperature. The reaction product was separated in the usual way into acidic and neutral fractions. The latter crystallized immediately on addition of a little methanol (38.3 mg., m.p. 149–151°). The product was recrystallized from 90 per cent ethanol, from which it formed rosettes of shiny rods melting at 150–151°; $[\alpha]_D^{24} = +34.9°$ (0.99 per cent in ethanol).

Analysis—$C_{29}H_{44}O_7$. Calculated, C 69.02, H 8.83; found, C 69.26, H 8.91

The diacetoxy keto ester can be conveniently obtained also by direct oxidation of appropriate chromatographic fractions containing its precursor VIII. Thus treatment of 700 mg. of Fraction E with 120 mg. of chromium trioxide in 10 cc. of acetic acid yielded 97 mg. of the pure compound.

The keto group in XIV is unreactive to 2,4-dinitrophenylhydrazine.

3(α),11(α)-Dihydroxy-12-ketocholanic Acid (XV)—The ester XIV (23.6

mg.) was dissolved in 2 cc. of 0.5 N methanolic potassium hydroxide solution, and the mixture allowed to stand at room temperature for 48 hours. The hydrolyzed product, recovered by ether extraction, crystallized immediately on addition of a few drops of ethyl acetate. Recrystallized from the same solvent, the acid formed solid triangular or rhombohedral blocks (16 mg.) with a melting point of 191–193°, which remained unchanged on further recrystallization; $[\alpha]_D^{22} = +84.9°$ (0.64 per cent in ethanol).

Analysis—$C_{24}H_{38}O_4$. Calculated, C 70.88, H 9.42; found, C 70.71, H 9.60

When 71 mg. of ester XIV were hydrolyzed by boiling with 2.5 cc. of 5 per cent methanolic potassium hydroxide, the crude product, on recrystallization from ethyl acetate, yielded inhomogeneous material (56 mg., m.p. 173–194°). By repeated recrystallization from the same solvent and finally from 95 per cent ethanol, 11 mg. of prisms melting at 201–203° were obtained; $[\alpha]_D = +72.8°$ (0.80 per cent in ethanol). A mixture with a sample of $3(\alpha),12(\alpha\,?)$-dihydroxy-11-ketocholanic acid (I) (m.p. 202–203°, $[\alpha]_D = +65.2°$ in ethanol) melted at the same temperature.

Analysis—$C_{24}H_{38}O_5$. Calculated, C 70.88, H 9.42; found, C 70.64, H 9.54

SUMMARY

The Wolff-Kishner reduction of $3(\alpha),12(\alpha\,?)$-dihydroxy-11-ketocholanic acid with sodium ethylate and hydrazine has been reinvestigated. The acid $C_{24}H_{38}O_3$ obtained in a previous study (1) has been identified as $3(\alpha)$-hydroxy-Δ^{11}-cholenic acid. The remainder of the reduction product has been shown to consist largely of three stereoisomeric $3(\alpha),11,12$-trihydroxycholanic acids which could be separated in the form of acetylated esters. In two of the stereoisomers, one of the hydroxyl groups in Ring C is not amenable to mild acetylation, while the third isomer can be completely acetylated under these conditions. It has been demonstrated that in one of the acids resistant to complete acetylation, the hindered hydroxyl group is in position 11; in the other, in position 12. The spatial orientation of the 11- and 12-hydroxyl groups in the three isomeric acids is discussed, and configurations are provisionally assigned.

The microanalyses reported in this paper were carried out by Mr. J. F. Alicino of this Division.

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THE WOLFF-KISHNER REDUCTION OF 3(α)-HYDROXY-11,12-DIKETOCHOLANIC ACID

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In a previous study (1) it has been shown that the Wolff-Kishner reduction of 3(α),12(α ?)-dihydroxy-11-ketocholanic acid (Marker-Lawson acid), formerly thought to be a 3(α),11-dihydroxy-12-keto acid, yields 3(α)-hydroxy-Δ^{11}-cholenic acid and three stereoisomeric 3(α),11,12-trihydroxycholanic acids, but none of the desired 3(α),11-dihydroxycholanic acid. At the time when these results were obtained the true structure of the starting acid had not yet been recognized, and the inertness of the keto group and its failure to undergo normal reduction to a methylene group was ascribed to steric hindrance by the adjacent hydroxyl group, then supposed to be in position 11. The study was therefore extended to 3(α)-hydroxy-11,12-diketocholanic acid, on the presumption that an enolic hydroxyl in that position might be spatially so situated as not to interfere with the normal reduction of the 12-keto group. The diketo acid had been previously described by Longwell and Wintersteiner (2) in the form of its 3-acid succinyl methyl ester (IV). The absorption spectrum of this derivative indicates that it exists entirely in the diketo form, whereas the free acid which we have now prepared from it by hydrolysis with alkali represents a stable enol form (V). This is entirely analogous to the behavior of the 11,12-diketocholanic acid of Wieland and Pasternak (3), whose conclusions regarding the structure of the two forms were confirmed spectrographically by Barnett and Reichstein (4). Since under the conditions of the Wolff-Kishner reaction immediate enolization could be anticipated, the succinoxy ester was used directly for the reduction with sodium ethylate and hydrazine. The non-crystalline reaction product was converted into acetylated methyl esters and the latter were fractionated chromatographically. Two crystalline products were obtained: (1) material melting at 124–126° which was similar in appearance and properties to corresponding chromatographic fractions of somewhat lower melting point occasionally obtained in the reduction of 3(α),12(α ?)-dihydroxy-11-ketocholanic acid (1). This product was not further investigated, but probably consists mainly of methyl 3(α)-acetoxy-Δ^{11}-cholenate, mixed with some of the isomeric $\Delta^{9,11}$ ester and possibly of methyl 3-acetoxycholanate (1); (2) an ester, $C_{27}H_{44}O_5$, melting at 146–148° which is identical with the

methyl 3(α)-acetoxy-11-hydroxycholanate (VIII) obtained by Lardon and Reichstein (5) from methyl 3(α)-acetoxy-Δ^{11}-cholenate via methyl 3(α)-

I. R = H
II. " = COCH$_2$CH$_2$COOH
III. " = COCH$_2$CH$_2$COOCH$_3$

IV. R = COCH$_2$CH$_2$COOH

V. R = R$_1$ = H
VI. " = CH$_3$, R$_1$ = H
VII. " = CH$_3$, " = CH$_3$CO

(VIII)

(IX)

acetoxy-11-ketocholanate (IX). Oxidation of the isolated compound to the latter ester confirmed its structure.

It is now evident from the work of Gallagher and his collaborators (6, 7) on the Wolff-Kishner reduction of the true $3(\alpha)$, 11-dihydroxy-12-ketocholanic acids that the 11-hydroxyl group in neither epimeric configuration prevents the normal reduction of the 12-keto group. This, of course, accounts also for the ready elimination of this group from the enolized diketone. It is furthermore well established by the work on the Marker-Lawson acid (1, 8) that an 11-keto group is not reduced in this manner, but instead is transformed into carbinol, in all probability via an enol form. The formation of a $3(\alpha)$, 11-dihydroxycholanic acid from the 11, 12-diketo acid is thus readily explained. The unsaturated acids simultaneously formed obviously arise by dehydration either from this dihydroxy acid, or from its 11-epimer, which may be more unstable under the conditions employed.

Lardon and Reichstein (5) have tentatively assigned the $11(\alpha)$ configuration to the acetoxy ester VIII. Their argument on this point is mainly based on the fact that the corresponding 3-desacetoxy compound, methyl 11-hydroxycholanate, can also be obtained by high pressure hydrogenation of methyl $11(\alpha)$, $12(\alpha)$-oxidocholanate (9). However, the possibility of a Walden inversion in the latter reaction was admitted. The 11-hydroxyl group in both methyl $3(\alpha)$-acetoxy-11-hydroxycholanate and methyl 11-hydroxycholanate is considered comparatively resistant to acetylation by the Swiss investigators. Experimental data were given only for the latter compound, and they show that heating with pyridine and acetic anhydride at 100° for 3 hours yielded an amorphous acetate besides some starting material. On the other hand, Long and Gallagher (6) report that the methyl $3(\alpha)$, 11-dihydroxycholanate which they obtained by Wolff-Kishner reduction of $3(\alpha)$, $11(\beta)$-dihydroxy-12-ketocholanic acid hydrazide hydrazone was readily converted into a diacetyl methyl ester (m.p. 118–119°) by heating with these reagents for 1 hour only. Since in some of the stereoisomeric 3, 11, 12-trihydroxycholanic acids the 11-hydroxyl group in the α configuration is amenable to acetylation under even milder conditions (1, 8), they assigned this configuration also to their $3(\alpha)$, 11-dihydroxycholanic acid. (This requires the assumption that epimerization at C_{11} has occurred under the influence of hot alkali before reduction of the 12-hydrazone group.) We therefore subjected material recovered from the mother liquors of the monoacetate VIII (m.p. 138–143°) to acetylation under the conditions used by Long and Gallagher, but the melting point remained unchanged. It therefore appears that the $3(\alpha)$, 11-dihydroxycholanic acid of these authors differs from the ester VIII by epimerism at C_{11}, and that the latter should be accorded the $11(\beta)$ configuration by the criterion adopted by Gallagher and ourselves (1, 8).

One of the disadvantages of the procedure described here is the consider-

able loss of material which attends the monosuccinylation of the starting ester I and the isolation of the diketo ester acid succinate IV after the oxidation step. Longwell and Wintersteiner (2) secured the latter compound without isolating the intermediate 3-acid succinate II of the starting ester. In the hope that by carrying out the two steps separately the overall yield could be improved, II has now been isolated and characterized. However, the losses in its purification are such that the use of the pure compound (m.p. 142–143°) for the subsequent oxidation is impracticable. For further characterization the dimethyl ester III (m.p. 86–88°) was prepared.

Though the oxidation reaction seems to proceed smoothly and rapidly, the purification of the resulting methyl 3-succinoxy-11,12-diketocholanate (IV) was beset with difficulties. The melting points were unsharp and lower than that (194–196°) previously reported (2). Nevertheless, the preparations were analytically pure, and showed the same ultraviolet absorption characteristics as the preparation of Longwell and Wintersteiner. In view of the generally unsatisfactory properties of the acid succinates, alternative methods for the preparation of the diketo acid, such as partial oxidation of the starting acid itself, in analogy to the preparation of 3(α)-hydroxy-12-ketocholanic acid from desoxycholic acid (10), or oxidation of methyl 3(α)-acetoxy-11(β)-hydroxy-12-ketocholanate (1), would appear preferable.

The 12-keto group in the succinoxy ester IV is reactive, as shown by the formation of a hydrazone hydrazide on treatment with hydrazine. The hydrolysis of IV to the enolic acid V, 3(α),11-dihydroxy-12-keto-$\Delta^{9,11}$-cholenic acid, is best accomplished by methanolic potassium hydroxide at room temperature. The enolic character of V (m.p. 165–167°) is evidenced by the strongly positive, blue-green ferric chloride reaction, and the intense light absorption around 280 mμ characteristic for cyclic α,β-unsaturated α-hydroxy ketones. The molecular extinction coefficient ϵ was somewhat lower than that observed by Barnett and Reichstein (4) for 11-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (7000 instead of 8700). The attempted preparation of the oxime and semicarbazone yielded inhomogeneous products. For further characterization the methyl ester VI and the 3,11-diacetoxy ester VII were prepared. They gave correct analytical values, but had unsharp and erratic melting points. The spectrum of the methyl ester VI has the same characteristics as that of the free acid, but acetylation of the enolic hydroxyl group (in VII) results in a displacement of the characteristic 280 mμ band to 243 mμ, and in an increase of its intensity. Similar shifts accompany the acetylation of the diosphenol forms of cholestane-2,3-dione (11) and cholestane-3,4-dione (12).

EXPERIMENTAL

3-Acid Succinate of Methyl 3(α),12(α ?)-Dihydroxy-11-ketocholanate (II)—A solution of 5 gm. of the methyl ester I and 11.8 gm. of succinic anhydride in 150 cc. of dry pyridine was allowed to stand at room temperature for 3 days, then boiled for 1 hour, and freed from most of the solvent by distillation *in vacuo*. The residual solution was poured into ice water, and the precipitated material extracted twice with 500 cc. of ether. The ether solution was washed several times with dilute hydrochloric acid and then extracted with three 200 cc. portions of 1 N potassium carbonate solution. The latter was acidified and extracted with ether. The dried ether solution on evaporation yielded about 4 gm. of a crystalline mass (m.p. 126–135°) which after two recrystallizations from small volumes of 90 per cent methanol weighed 2.4 gm. and melted at 139–143°. On further recrystallization of a small sample from 60 per cent ethanol platelets which melted at 142–143° after sintering at 140° were obtained. However, with some other batches the melting point remained unsharp at 138–142°, and could not be improved by changing the solvent used for recrystallization (ether-hexane).

Analysis—$C_{29}H_{44}O_8$. Calculated, C 66.88, H 8.52; found, C 66.43, H 8.36

11.91 mg. required 2.24 cc. of 0.01 N NaOH for neutralization; calculated, 2.29 cc.

Treatment of 52 mg. of the succinate with an ethereal solution of diazomethane afforded the dimethyl ester III, which after two recrystallizations from methanol melted at 84–86°.

Analysis—$C_{30}H_{45}O_8$. Calculated, C 67.37, H 8.68; found, C 67.54, H 8.85

3-Acid Succinate of Methyl 3(α)-Hydroxy-11,12-diketocholanate (IV)—4.4 gm. of the crude succinate II (m.p. 126–135°) were dissolved in 40 cc. of acetic acid (distilled over chromium trioxide). The solution was cooled to near ice temperature, and 621 mg. of chromium trioxide (equivalent to 1.1 atoms of oxygen) dissolved in 5 cc. of cold 90 per cent acetic acid were added in small portions in the course of 10 minutes. On standing at room temperature a crystalline precipitate formed, which after 1 hour was separated from the purple-green supernatant by filtration, washed with cold 30 per cent ethanol, and dried. The crystalline product (1.25 gm., m.p. 182–188°) was recrystallized several times from 90 per cent ethanol, whereby it yielded 897 mg. of fine platelets with a constant melting point of 188–193°. The compound is fairly soluble in cold ethanol and acetone, but sparingly soluble in ether; $[\alpha]_D^{24} = +106°$ (1.15 per cent in acetone).

Analysis—$C_{21}H_{42}O_3$. Calculated, C 67.16, H 8.16; found, C 67.01, H 8.14

The absorption spectrum in ethanol showed a maximum at 284 mμ (ϵ = 140) and a minimum at 250 mμ (ϵ = 65).

The filtrate from the crude crystals was brought to dryness *in vacuo* and the residue distributed between water and ether containing 10 per cent of acetone. Three extractions with 450 cc. of this solvent mixture were necessary to bring all the material into solution. The combined ether solutions were thoroughly washed with water, dried, and evaporated. By recrystallization of the residue 830 mg. of the ester (m.p. 186–191°) were recovered.

The succinate IV differs from the unoxidized compound II by its low solubility in ether, and by its capacity to form an insoluble crystalline sodium salt when its ethereal solution is distributed with aqueous sodium carbonate. However, attempts to utilize these properties for purification did not result in improved yields or preparations with sharper melting points.

A solution of partially purified succinate IV (200 mg., m.p. 181–186°) in 5 cc. of absolute ethanol containing 0.5 cc. of 85 per cent hydrazine hydrate was boiled for 17 hours. The crystals which separated on cooling (42 mg., m.p. 165–167.5°) were collected and recrystallized from 95 per cent alcohol. They were identified by analysis as succinic acid dihydrazide.

Analysis—$C_4H_{10}O_2N_4$. Calculated. C 32.85, H 6.90, N 38.3
                    Found.   " 33.60, " 7.08, " 38.7

The filtrate and washings were evaporated *in vacuo* and a few cc. of water were added to the crystalline residue. After standing overnight in the refrigerator, the crystals were filtered off and washed with cold water. The product (131 mg.) melted over a range of 140–170°. It was recrystallized twice from 25 per cent ethanol and then twice from 50 per cent methanol. The platelets thus obtained (58 mg.) melted with gas evolution at 150° after sintering at 144°. According to the analysis the compound must be the 12-hydrazone of 3(α)-hydroxy-11,12-diketocholanic acid hydrazide.

Analysis—$C_{24}H_{40}O_3N_4$. Calculated. C 66.61, H 9.33, N 12.96
                    Found.   " 67.20, " 9.34, " 12.47

The fact that the compound is derived from the diketo form of the acid follows from the negative ferric chloride reaction and from the ultraviolet absorption characteristics (maximum at 301 mμ, ϵ = 238; minimum at about 245 mμ, ϵ = 100; the exact position of the minimum is obscured by several very small secondary maxima at 243, 250, 256, and 263 mμ). The

shift of the main band from its position in IV towards the red end of the spectrum and the increase in its intensity is comparable to the "semi-carbazone shift" observed with α,β-unsaturated ketones.

3(α),11-Dihydroxy-12-keto-$\Delta^{9,11}$-cholenic Acid (V)—812 mg. of the succinate IV (m.p. 187–191°) were dissolved in 30 cc. of methanolic 0.5 N potassium hydroxide solution. After standing at room temperature for 48 hours, the mixture was separated in the usual manner into neutral and acidic fractions. The acidic material (648 mg.) was dissolved in a small amount of absolute ether, from which it crystallized slowly in the form of large prisms. Recrystallization from 50 per cent ethanol yielded 497 mg. melting at 165–167° after slight sintering at 160°. Further recrystallization did not change the melting point. An ethanolic solution of the acid gave the characteristic blue-green ferric chloride reaction described for 11-hydroxy-12-keto-$\Delta^{9,11}$-cholenic acid (3; 4); $[\alpha]_D^{23} = +91.1°$ (1.0 per cent in ethanol).

Analysis—$C_{24}H_{36}O_5$. Calculated, C 71.24, H 8.97; found, C 71.16, H 9.12

The absorption spectrum in ethanol showed a single maximum at 281 mμ ($\epsilon = 7000$).

Methyl 3(α),11-Dihydroxy-12-keto-$\Delta^{9,11}$-cholenate (VI)—The neutral fraction from the hydrolysis experiment yielded needle-shaped crystals melting at 57–70°, which could not be effectively purified. Since they gave a strong ferric chloride test and showed the same absorption spectrum as V, they undoubtedly consisted for the most part of the methyl ester VI of the enolized acid. A somewhat better preparation of this ester was obtained by treating the acid V (105 mg.) with a slight excess of diazomethane in ether (to persistence of a faint yellow color). The residue of the ether solution was taken up in 1 cc. of ether. Addition of 3 cc. of pentane and seeding with the crystals from the hydrolysis experiment caused immediate crystallization. The apparently homogeneous needles thus obtained (92 mg.) melted at 68–75°. The melting point was not materially changed by two subsequent recrystallizations from the same solvents; $[\alpha]_D^{22} = +101°$ (0.91 per cent in ethanol).

Analysis—$C_{25}H_{38}O_5$. Calculated, C 71.72, H 9.15; found, C 71.55, H 9.41

The absorption spectrum showed the maximum at 280 mμ characteristic for the enol form ($\epsilon = 5200$).

In another experiment in which the acid was allowed to react with a slight excess of diazomethane at 4° for 12 hours platelets were obtained which after recrystallization melted at 80–122°. The unsatisfactory melting point properties of the compound may be due to the presence of some of the diketo form, as indicated by the low ϵ, and of small amounts of the

11-methoxy methyl ester formed by the participation of the enolic hydroxyl group in the reaction with diazomethane.

Methyl 3(α),11-Diacetoxy-12-keto-Δ⁹,¹¹- cholenate (VII)—60 mg. of the crude methyl ester obtained by methylation at 4° were treated with 1 cc. of pyridine and 0.5 cc. of acetic anhydride at room temperature. After 3 days the mixture was worked up in the usual way. The crude product (63 mg., m.p. 118–136°) was recrystallized twice from ether-pentane, from which it formed rosettes of platelets melting at 123–128°.

Analysis—$C_{29}H_{42}O_7$. Calculated, C 69.28, H 8.43; found, C 69.20, H 8.49

Another independently prepared specimen melted at 161° after sintering at 120°; found, C 69.61, H 8.48.

The absorption spectrum in ethanol showed a single maximum at 243 mμ (ϵ = 7800).

Methyl 3(α)-Acetoxy-11-hydroxycholanate (VIII)—A mixture of 1.5 gm. of partially purified succinate IV (m.p. 182–188°), 1.5 gm. of sodium, 20 cc. of absolute ethanol, and 1.5 cc. of hydrazine hydrate was heated in a bomb tube at 200° for 6 hours. The solution was diluted with water, acidified, and extracted with ether. The well dried residue of the ether solution was esterified with diazomethane and then acetylated with pyridine and acetic anhydride (room temperature, 24 hours). The neutral fraction (1.1 gm.) was dissolved in 50 cc. of benzene-hexane, 1:4, and adsorbed on a column (20 × 300 mm.) of aluminum oxide. The column was washed consecutively with 500 cc. each of (a) benzene-hexane, 1:4, (b) benzene-hexane, 1:1, (c) benzene, and (d) ether. The effluent solutions were collected in 50 cc. portions. The material eluted with mixture (a) was mostly crystalline and on recrystallization from methanol yielded 53 mg. of unsaturated esters melting at 124–126°. Eluates (b), (c), and (d) were amorphous, but most of the fractions in (c), together 286 mg., crystallized when they were dissolved in a little absolute ether, and hexane was added to turbidity. The crude crystals were collected (137 mg., m.p. 131–145°) and recrystallized from the same solvent mixture, yielding 90 mg. of rods melting at 146–148°. Further recrystallization did not change the melting point; $[\alpha]_D^{22} = +56.1°$ (0.945 per cent in chloroform).

Analysis—$C_{27}H_{44}O_5$. Calculated, C 72.28, H 9.89; found C 72.39, H 9.80

Methyl 3(α)-Acetoxy-11-ketocholanate (IX)—The ester VIII (42 mg.) was dissolved in 1.8 cc. of acetic acid containing 18.5 mg. of chromium trioxide. The solution was allowed to stand 17 hours at room temperature and then worked up in the usual way. The crude product (41 mg., m.p. 128–131°) was recrystallized from ether-pentane, from which it formed

hexagonal platelets melting at 130–132°; $[\alpha]_D^{22} = +66.3°$ (0.91 per cent in acetone).

Analysis—$C_{27}H_{42}O_4$. Calculated, C 72.61, H 9.48; found, C 72.49, H 9.45

SUMMARY

The preparation, properties, and various derivatives of $3(\alpha)$-hydroxy-11,-12-diketocholanic acid are described. The free acid, obtained by alkaline hydrolysis of the corresponding 3-succinoxy methyl ester, exists actually in the enolic form, that is as $3(\alpha)$,11-dihydroxy-12-keto-$\Delta^{9,11}$-cholenic acid, whereas the succinoxy ester represents the diketo form.

The Wolff-Kishner reduction of this acid yielded besides unsaturated acids a $3(\alpha)$,11-dihydroxycholanic acid, isolated in the form of the methyl $3(\alpha)$-acetoxy-11-hydroxycholanate, previously obtained via a different route by Lardon and Reichstein (5).

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LETTERS TO THE EDITORS

EVIDENCE FOR THE EXISTENCE OF AN UNIDENTIFIED GROWTH STIMULANT IN PROTEINS*

Sirs:

Recently, Woolley and his associates have presented evidence for the existence in proteins of an unidentified component which stimulates the growth of certain microorganisms,[1] and which appears to improve the rate of gain of mice receiving a ration in which the nitrogen is furnished in the form of acid-hydrolyzed casein supplemented with cystine and tryptophane.[2] This substance has tentatively been given the name "strepogenin."[3]

For the past 3 years, investigations which may be related to those of Woolley have been under way in this laboratory. Attention has been directed toward discovering an explanation for the divergence in the growth rate of animals (rats) upon diets containing (1) mixtures of purified amino acids and (2) native proteins.

Considerable improvement over the growth observed in our earlier studies has been induced by the use of more adequate vitamin supplements than were available previously, and by certain alterations in our basal rations. However, even with these modifications, the gains are distinctly inferior to those induced uniformly by rations containing proteins of superior quality. This is exemplified by the fact that the average gain in 28 days of forty-seven male weanling rats receiving a diet containing 18 per cent of casein plus 0.2 per cent of *dl*-methionine was 143 ± 1.1 gm., while the average gain for the same period of 152 male rats upon a diet carrying nineteen amino acids was 97 ± 0.7 gm. The accompanying table summarizes the effects exerted by replacing part of the amino acid mixture by an equal weight of whole or hydrolyzed protein. The total gains are to be compared with the figure (97 ± 0.7 gm.) given above.

[1] Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, **67**, 1734 (1945).

[2] Woolley, D. W., *J. Biol. Chem.*, **159**, 753 (1945).

[3] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).

Supplement	Amount in diet	No. of rats	Average total gain in 28 days with P.E.M.
	per cent		*gm.*
Whole casein.	5	10	126 ± 2.5
Acid-hydrolyzed casein*..............	5	7	105 ± 2.9
" " *..............	10	7	114 ± 3.1
Whole fibrin.........................	5	58	123 ± 0.9
Acid-hydrolyzed fibrin†..............	5	30	117 ± 1.0

* Hydrolyzed for 16 hours.
† Hydrolyzed for 4 hours.

Although in some tests the number of animals was small, the results are sufficiently clear cut to demonstrate that the inclusion of 5 per cent of either casein or fibrin in the diet exerts a distinct effect upon growth. On the other hand, complete hydrolysis with acid (16 hours) destroys most of the activity, while partial hydrolysis (4 hours) is not so detrimental.

Attempts to concentrate the active material have yielded extremely variable results. In some instances, active fractions have been obtained from incompletely hydrolyzed proteins; in others, for no obvious reasons, apparently identical methods have yielded negative findings. Tests have been applied to seventeen additional proteins and protein-rich foods. All materials examined have shown activity, with the possible exception of oxyhemoglobin from sheep blood, but none was more potent than casein and fibrin.

These findings are believed to point to the presence in proteins of an unidentified substance which, like arginine, is not necessary for fairly rapid gains, but is required for maximum increases in weight. Whether the substance is identical with strepogenin remains to be established. Our investigations are being continued.

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 Noyes Laboratory of Chemistry   WILLIAM C. ROSE
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 Urbana

THE NUTRITION OF LACTOBACILLUS GAYONII

Sirs:

Recent work of Cheldelin, Riggs, and Sarett[1] on the nutrition of *Lactobacillus gayonii* 8289 indicated that an essential factor of unknown composition was necessary for growth of this organism.

On a basal medium, containing the known essential and stimulatory factors required by the lactic acid bacteria, we have found that the factor is merely stimulatory in nature.

In preliminary work on the isolation of the factor it was noted that there was marked destruction of activity by such commercial enzyme preparations as taka-diastase or clarase. The pH optimum of the inactivation was 4.0. This suggested that the active enzyme could be a phosphatase and that inactivation was proceeding by liberation of phosphorus from the

TABLE I

Amounts of Substances Necessary to Promote Half Maximum Growth of Lactobacillus gayonii

Hydrolyzed yeast nucleic acid	Adenylic acid	Guanylic acid	Uridylic acid	Cytidylic acid	Adenylic, guanylic, uridylic, and cytidylic acids
γ per 10 ml.	γ per 10 ml.	γ per 10 ml.	γ per 10 ml.	γ per 10 ml.	γ each per 10 ml.
82	300	212	168	150	38

factor. As the most likely series of compounds would be the nucleotides, an ammonia-hydrolyzed preparation of yeast nucleic acid[2] was tested for its ability to support growth of *Lactobacillus gayonii*. Such a preparation was found to be highly active.

The results with the hydrolyzed yeast nucleic acid, the nucleotides, and a combination of the nucleotides are presented in Table I. Any one of the four nucleotides is able to support growth of *Lactobacillus gayonii*. The most active compound is cytidylic acid. The nucleotides are less active than the hydrolyzed nucleic acid. This suggests that the nucleic acid contains some factor that makes the organism more sensitive to the nucleotides.

Only one nucleoside was available for comparison. Guanosine was found to be inactive. Further evidence for the inactivity of the nucleosides was obtained by incubating hydrolyzed yeast nucleic acid or the nucleotides with taka-diastase. Representative data for hydrolyzed yeast nucleic

[1] Cheldelin, V. H., Riggs, T., and Sarett, H. P., *Federation Proc.*, **4**, 85 (1945).

[2] Levene, P. A., *J. Biol. Chem.*, **33**, 425 (1918).

acid, adenylic acid, and uridylic acid are presented in Table II. The ratio of the per cent phosphorus liberation to per cent inactivation is approximately constant and nearly equal to unity. This concomitant inactivation

TABLE II

Enzyme Inactivation of Hydrolyzed Yeast Nucleic Acid, Adenylic Acid, and Uridylic Acid

Substrate	Taka-diastase added	Incubation	P liberated	Inactivation	Per cent P liberated / Per cent inactivation
	mg.	*mg.*	*hrs.*	*per cent*	*per cent*	
Hydrolyzed	10	None	24	None	None	
yeast nucleic	10	20	6	38.31	46.0	0.83
acid	15	20	6	47.7	54.4	0.88
	15	20	24	70.68	78.4	0.90
	15	50	24	79.13	85.1	0.93
Adenylic acid	8	40	6	31.77	28.06	1.13
	8	40	24	95.95	97.7	0.98
Uridylic acid	8	40	6	76.7	78.58	0.98
	8	40	24	100.0	99.0	1.01

with phosphorus liberation, taken in conjunction with the inactivity of guanosine, strongly suggests that the nucleosides are inactive for this organism.

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                                                                   E. BOGGIANO

SUCCINIC DEHYDROGENASE OF MAMMALIAN LIVER

Sirs:

In the course of a series of experiments designed to determine the distribution within the normal and malignant cell of a number of enzymes, use has frequently been made in this laboratory of a neutral saline extract containing the cytoplasmic components of mammalian liver cells. The preparation and fractionation of the "cytoplasmic extract" have been carried out according to the method of Claude.[1] In the case of rat liver[2] it was found that approximately 70 per cent of the cytochrome oxidase and succinoxidase activity of the extract could be recovered in the large granule or mitochondrial fraction (sedimentable at 2700 $\times$ g for 20 minutes). A smaller particulate component (sedimentable at 18,000 $\times$ g for 90 minutes) had very little activity, and the remaining soluble material of the extract had none.

Further studies of the succinoxidase present in the mitochondrial fraction showed that the activity of the system, as determined aerobically with added cytochrome c, declined rapidly when the granules were suspended in hypotonic media (0.01 M $NaHCO_3$), whereas the succinic dehydrogenase activity, as determined anaerobically by the ferricyanide technique,[3] declined slowly. If the granules were repeatedly washed with hypotonic media, however, the anaerobic activity could also be reduced to low levels. It was noted that the soluble material obtained by the initial treatment of the granules with 0.01 M $NaHCO_3$ consistently possessed faint but definite succinic dehydrogenase activity. The latter finding suggested that the dehydrogenase is a soluble enzyme but bound too firmly to particulate material to be separated by simple aqueous extraction.

After a number of attempts it has been found possible to obtain clear solutions showing fairly high succinic dehydrogenase activity by the following procedure. The mitochondrial fraction of guinea pig liver is isolated, washed once with 0.85 per cent NaCl containing 0.025 M $NaHCO_3$, and firmly packed by centrifugation at 18,000 $\times$ g for 1 hour. The granules are then treated twice with cold acetone, dried quickly *in vacuo*, and extracted for 18 hours at 0° with 0.01 M $NaHCO_3$. The clear yellow solutions obtained by centrifugation of the bicarbonate extract mixtures at 18,000 $\times$ g for 1 hour have shown $Q_{CO_2}^{N_2}$ values of 150 to 300 by the ferricyanide method with succinate as substrate. It is estimated that the amount of

[1] Claude, A., in Biological symposia, Lancaster, **10**, 111 (1943). Claude, A., *J. Exp. Med.*, in press.

[2] Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., unpublished experiments.

[3] Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **32**, 936 (1938).

dehydrogenase which can be obtained in solution by this method represents a yield of approximately 10 per cent of the activity originally present in the granules.

Preliminary studies of the soluble succinic dehydrogenase indicate that the enzyme can be precipitated with $(NH_4)_2SO_4$ between 20 and 50 per cent saturation with very little loss. When redissolved in 0.01 M $NaHCO_3$, this $(NH_4)_2SO_4$ fraction retained 90 per cent of its original activity after standing for 4 days at 0° and was not affected by 18 hours dialysis against 0.01 M $NaHCO_3$. Cytochrome oxidase is apparently not present either in the bicarbonate extract or in the dialyzed $(NH_4)_2SO_4$ fraction, since both solutions reduce cytochrome c in the presence of air. The rate of reduction of cytochrome c is not accelerated, however, by the addition of succinate.

Further studies of the succinic dehydrogenase present in the mitochondrial fraction of mammalian liver will be reported in the near future.

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THE DEMETHYLATION OF N¹-METHYLNICOTINAMIDE AND THE INFLUENCE OF THE METHYL GROUP ON THE FATTY LIVER OF RATS [1]

Sirs:

Control rats of comparable age and weight were fed the Griffith and Wade diet[1] with glycocyamine, 10 mM per kilo of diet. Experimental rats were fed N¹-methylnicotinamide in addition. It was anticipated that demethylation would result in the release of nicotinamide, which would be reflected in a high urinary output of the vitamin. Furthermore, creatine and creatinine formation might be enhanced and would be similarly excreted. Lastly, the available methyl group would prevent or diminish fat deposition in the livers of rats fed such a diet.

Rat No.	Diet supplement	Liver fat (wet weight)	Average
		per cent	*per cent*
1	Glycocyamine	36.1	
2	"	39.1	
3	"	39.7	
4	"	34.7	37.4
5	" N¹-methylnicotin-amide, 2%	24.5	
6	Glycocyamine,N¹-methylnicotin-amide, 2%	8.2	
7	Glycocyamine,N¹-methylnicotin-amide, 2%	10.6	
8	Glycocyamine,N¹-methylnicotin-amide, 2%	23.5	16.7

Eight adult rats (litter mates) were placed in metabolism cages. Four ingested the diet with glycocyamine. The other four, after a control period of 10 days, were given in addition 1 per cent N¹-methylnicotinamide incorporated in the diet. The daily urinary output of nicotinic acid in the first group averaged 4.5 γ per rat per day throughout the experiment. In the second group the level rose from an average of 3.5 γ during the control period to 11.8 γ after N¹-methylnicotinamide ingestion. This persisted for the 3 months of the experiment. There was no noticeable difference in the urinary output of creatine and creatinine between the two groups throughout the experiment. When 1 per cent methionine was used instead of N¹-methylnicotinamide on a group of eight rats similarly treated,

[1] Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **132**, 627 (1940).

the creatine and creatinine output was similarly unaffected.   The excretion of these two substances, therefore, was not a useful criterion.

To test the effect on fatty livers, eight 3 week-old litter mates were used.   Four ingested the diet with glycocyamine.   The remaining four were fed in addition 2 per cent N^1-methylnicotinamide.   The rats were sacrificed 30 days after the beginning of the experiment for liver fat analysis.

The table shows that rats fed N^1-methylnicotinamide averaged less than half the amount of fat found in the controls.   This is particularly pronounced in two of the rats.

These results suggest that N^1-methylnicotinamide is demethylated with the release of nicotinamide.   This explains our finding[2] that the compound possesses antipellagra activity in the dog.   The effect on fat deposition in the liver suggests that the methyl group is biologically active.   Further confirmation with a large group of rats is in progress.

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[2] Najjar, V. A., Hammond, M. M., English, M. A., Wooden, M. B., and Deal, C. C., *Bull. Johns Hopkins Hosp.*, **74,** 406 (1944).

A COMMON FACTOR IN THE ENZYMATIC ACETYLATION OF SULFANILAMIDE AND OF CHOLINE*

Sirs:

The enzymatic condensation of sulfanilamide and acetate, with adenyl pyrophosphate as an energy donor, had been found dependent on a heat-stable coenzyme.[1] Recently, by fractionation of pork liver, a relatively concentrated preparation of this coenzyme was obtained, about 100 times the strength of the original boiled liver extracts. During the process of purification we became increasingly aware of the great similarity between our acetylation system in liver and the one, described by Nachmansohn and Machado,[2] in the brain extracts which combines choline with acetate, with adenyl pyrophosphate again acting as condensing agent.

It seemed likely then that the coenzyme, active in the acetylation in liver, might also be a factor in acetylation in brain. The observations of Nachmansohn and his coworkers[2,3] on partly reversible inactivation by various mild procedures were furthermore suggestive. It was therefore decided to test our purified coenzyme on the acetylation system in brain. Very active enzyme extracts were prepared for this purpose by extraction of acetone brain powder[3,4] (pigeon). Reversible inactivation was obtained either by autolysis for 20 hours at 5° or by dialysis against 0.1 M potassium chloride solution for 2 hours at room temperature. The latter procedure gave more consistent results and it was used in the following experiment.

Each tube contained 0.5 ml. of dialyzed and thoroughly centrifuged extract of pigeon brain, corresponding to 25 mg. of acetone brain powder, in a total volume of 1.0 ml. The final concentration of additions was 0.01 M sodium fluoride, 0.004 M choline chloride and sodium acetate, 0.01 M sodium citrate, 0.015 M cysteine, 0.005 M sodium adenyl pyrophosphate, and 0.5 mg. per ml. of eserine. The tubes were incubated for 70 minutes at 37°.

Coenzyme added, γ	0	13	26	104	208
Acetylcholine formed per gm. acetone powder, γ	200	1010	1120	1440	1360

Acetylcholine was determined by the frog rectus method of Chang and Gaddum[5] in the manner described by Nachmansohn and Machado.[2]

[1] Lipmann, F., *J. Biol. Chem.*, **160**, 177 (1945).
[2] Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397 (1943).
[3] Nachmansohn, D., and John, H. M., *J. Biol. Chem.*, **158**, 157 (1945).
[4] Feldberg, W., and Mann, T., *J. Physiol.*, **104**, 8 (1945).
[5] Chang, H. C., and Gaddum, J. H., *J. Physiol.*, **79**, 255 (1933).

It appears that the coenzyme which we had isolated, using as a test system the acetylation of sulfanilamide in liver, is likewise a coenzyme for the choline-specific acetylation system in brain. In addition to the coenzyme, both systems require cysteine, citrate, or both[2,3] for full activation. By comparison with similar systems we assume that our best coenzyme preparation may contain 5 to 10 per cent of the pure substance. The present preparation contained 13.6 per cent nitrogen and 5.7 per cent phosphorus.

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ISOLATION OF ANDROSTANE-3 (α), 11-DIOL-17-ONE FROM THE URINE OF NORMAL MEN

Sirs:

Isolation of androstane-3 (α), 11-diol-17-one from the urine in cases of adrenal cortical tumor and adrenal cortical hyperplasia[1] stimulated interest in the possible occurrence of this substance in normal urine. A pool of 105 liters of urine was collected from six normal men 30 to 46 years of age. The urine was collected in 5 gallon bottles containing 90 ml. of concentrated hydrochloric acid and 300 ml. of toluene. It was processed as previously described. The ketonic fraction was dissolved in benzene and chromatographed on a column of alumina (Merck, Rahway, New Jersey, standardized according to Brockmann). The various fractions were eluted with benzene and with benzene containing increasing amounts of isopropyl alcohol. The fraction eluted with benzene containing 0.02 volume of isopropyl alcohol weighed 31 mg. after recrystallization from acetone and ethyl acetate and melted at 196–198°; $[\alpha]_D^{26} = +94.8° \pm 3°$. A mixture with androstane-3(α),11-diol-17-one (m.p. 197–198°; $[\alpha]_D^{26} = +96.6° \pm 2°$) melted at 196–198°. The acetate, prepared with acetic anhydride and pyridine, melted at 238–240°; the melting point was not depressed by admixture of androstane-3(α)-acetoxy-11-ol-17-one. Thus androstane-3(α),11-diol-17-one was isolated in the amount of 0.3 mg. per liter from the pooled urine of six normal men. This amount is considerably less than the amounts that were isolated (1.5 to 2.1 mg. per liter) from the urine in two cases of adrenal cortical tumor and four of adrenal cortical hyperplasia. In one case of tumor, however, the amount was 0.26 mg. per liter.

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[1] Mason, H. L., *J. Biol. Chem.*, **158**, 719 (1945). Mason, H. L., and Kepler, E. J., *J. Biol. Chem.*, **161**, 235 (1945).

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